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THE EFFECT OF IRRADIATION ON COBRA VENOM AND ANTIVENIN.

ALBERT EIDINOW, M.B., B.S.LOND.

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BACTERIA and filter-passing viruses suspended in protein-free solutions are rapidly destroyed when exposed to ultra-violet rays of wave-lengths shorter than 3100 Å.U. Further research has indicated that there is a selective variation in the sensitivity of these organisms to light. In general the toxins and antitoxins have a higher resistance to radiation than bacteria and unicellular organisms, but this may be due to proteins and glucosides associated with these substances, which, absorbing the short ultra-violet rays, protect the toxin and antitoxin molecules. The review of the earlier literature on the action of light on toxin and antitoxin does not clearly indicate the physical characters and intensity of the sources of light employed, and in many cases, description of experiments fail to prove that temperature effects were sufficiently controlled. A series of investigations were therefore carried out to study the effect of a standardized source of ultra-violet rays on solutions of cobra-venom and anti-venin.

TECHNIQUE.

Preparation of cobra venom and determination of the minimal lethal dose for rabbits and rats.—A sterile solution of cobra venom* was prepared by dissolving a weighed quantity of dried venom in sterile saline. Testing this solution *in vivo*, by intravenous injection into rabbits, it was found that 1 c.c. of a 1/2500 solution was the minimal lethal dose for rabbits weighing 1500–2000 gm. About one hour after injection these animals died with the symptoms of venom poisoning and demonstrated typical post-mortem changes. A similar dose of venom injected into the peritoneal cavity of rats weighing 60–80 gm. was fatal 30–60 minutes later.

* The specimen of cobra venom used was kindly given to me by Capt. S. R. Douglas.

The nature of the source of light.—The source of light employed for irradiation in these experiments was two quartz mercury vapour lamps (atmospheric type, 2·5 amp. D.C. and 110 volts between electrodes). These were placed side by side, and were 9 in. distant from the medium irradiated. The spectrophotographic examination of this source of light showed that rays from infra-red to 2200 Å.U. were emitted. Previous biological tests carried out under identical conditions of irradiation showed that (1) the lethal time for a culture of infusoria in water at 19° C., irradiated 9 in. distant, was 2 minutes; (2) a 2% suspension of washed red blood-corpuscles in saline was hæmolysed in 5 minutes; (3) a culture of staphylococci suspended in saline was sterilized after 3 minutes' exposure; (4) the minimal erythema dose on irradiating a patch of white skin on the forearm was 3½ minutes; (5) a standard solution of acetone methylene blue in a quartz tube was bleached 1° on the standard scale after 15 minutes' irradiation.

The technique of irradiation of solution of cobra venom.—The solution of cobra venom employed was placed in a sterile (fused silica) "quartz" flask (50 c.c. capacity). The flask was attached to the spindle of an electric motor by means of a sterile rubber cork. By these means the flask was rotated slowly in a horizontal position to the lamps, and a thin film of the solution of venom was constantly exposed to the source of light. The distance of the flask from the source of light was 9 in., and during rotation the flask was immersed in a bowl containing water at 18° C., so that the flask and contents were kept cool during irradiation.

TESTS *IN VIVO*.

Experiment No. 1.—Irradiation of Cobra Venom with Mercury Vapour Lamp (5700–2300 Å.U.).

5 c.c. of the solution of cobra venom (1/2500) were placed in the quartz flask and exposed to the source of light in the manner previously described. After 15, 30 and 60 minutes' irradiation, samples were removed and injected intravenously into rabbits. A lethal dose of the venom in saline was completely destroyed after 15 minutes' irradiation, and all animals injected with the irradiated venom survived, showing no ill-results from injection. Table I indicates the type of protocol of some of the results obtained:

TABLE I.

Rabbit No.	Weight in gm.	Dose of venom injected intravenously.	Time of irradiation; infra-red–2200 Å.U.	Dose per kgm. weight.	Result.
234	2040	1 c.c.	15 minutes	0·000196	Alive.
237	2040	1 „	30 „	0·000196	„
238	1750	2 „	60 „	0·000456	„
234	1850	1 „	*Nil*	0·000210	Died 50 minutes.

Similar experiments carried out by injecting 1 c.c. of cobra venom in saline (1/1250) into the peritoneal cavity of rats weighing 60–80 gm. showed that

the exposure for 15 minutes of the venom to rays from the mercury vapour lamp in a quartz flask destroyed its toxic action.

Experiment No. 2.—Exposure of Cobra Venom to Rays of Mercury Vapour Lamp Filtered through a Screen of Vita-glass (Infra-red–2800 Å.U.).

A screen of vita-glass (Chance's 1926, 2·4 mm. thick) was interposed between the source of light and the quartz flask, and the venom was exposed as in Experiment No. 1. The spectrophotographic examination of this screen showed that the rays from infra-red to 2800 Å.U. only were transmitted. All the animals injected with the venom irradiated through the vita-glass screen died. The post-mortem examination carried out directly after death showed that there was no evidence of intra-vascular clotting; the blood in the portal vein, inferior vena cava and right auricle was fluid. It therefore appears that irradiation of cobra venom with the rays from infra-red–2800 Å.U. destroys the hæmolysin and cytolysin, but leaves the neurotoxin unchanged (Table II).

TABLE II.

Rabbit No.	Weight in gm.	Dose of venom, 1/2500.	M.V.L. irradiation through vita-glass; infra-red—2800 Å.U.	Dose per kgm. weight.	Lethal time.	Result.
326	1750	1 c.c.	*Nil*	0·000228	40 minutes	Died.
327	1780	1 „	15 minutes	0·000227	45 „	„
328	1630	1 „	60 „	0·000245	55 „	„

Experiment No. 3.—Exposure of Cobra Venom in Saline as in Experiment No. 1 to Radiation from Quartz Mercury Vapour Lamp, but Filtered through a Screen of "Window-glass" 4 mm. thick (Radiations Infra-red–3300 Å.U.).

The screen of ordinary window-glass, 4 mm. thick, filtered off all rays of wave-length shorter than 3300 Å.U. All the animals injected with cobra venom

TABLE III.

Rabbit No.	Weight in gm.	Dose of cobra venom 1/2500 injected intra-venously.	Irradiation through window-glass; infra-red—3300 A.U.	Dose per kgm. weight.	Lethal. time.	Result.
242	1920	1 c.c.	...	0·000208	75 minutes	Died.
243	1880	1 „	15 minutes	0·000213	45 „	„
244	1880	1 „	30 „	0·000213	35 „	„
245	1880	1 „	60 „	0·000220	30 „	„

irradiated in this manner died, and showed typical symptoms and post-mortem changes and intra-vascular clotting (Table III).

From these experiments *in vivo* 1·0 c.c. of a solution of cobra venom diluted with sterile saline to a concentration of 1/2500 is a definite lethal dose for rabbits weighing 1500–2000 gm. and rats of 60–80 gm. The exposure of this solution to the rays emitted by a quartz mercury vapour lamp indicate that neurotoxin is only destroyed by ultra-violet rays shorter than 2800 Å.U., while the hæmolysin is destroyed by rays shorter than 3300 Å.U. In order to confirm these observations a series of experiments were carried out *in vitro*, to observe the hæmolytic action of cobra venom on a 5% suspension of rabbit's washed red blood-corpuscles in saline.

EXPERIMENTS *IN VITRO*.

Experiment No. 4.—Cobra Venom and Hæmolysis of a Suspension of Rabbit's Washed Red Blood-corpuscles.

A 5% suspension of rabbit's red blood-corpuscles in saline was prepared and the hæmolytic action of the solution of cobra venom was tested. In the presence of lecithin (1/5000 in saline), a solution of cobra venom 1/50,000 in saline completely hæmolysed the red blood-corpuscles. Cobra venom irradiated in a quartz flask completely lost its hæmolytic action after 5 minutes' irradiation. The hæmolysin was also destroyed when venom was irradiated through a vita-glass screen, but no destruction was observed with irradiation with the glass screen. These experiments confirm the previous results that the hæmolysis of cobra venom can be destroyed by ultra-violet rays of longer wave-length, *i. e.* 2800 Å.U., than those necessary to alter neurotoxin. The latter is only sensitive to rays shorter than 2800 Å.U. (Table IV).

TABLE IV.

Volume of 5% suspension of rabbit's R.B.C. in saline in c mm.	Volume of cobra venom 1/2500 in c.mm.	Volume of lecithin 1/5000 in c.mm.	Volume of saline in c.mm.	Total volume in c mm.	Concentration of venom.	Time of irradiation of venom with M.V.L. in minutes and resulting hæmolysis.						
						Nil.	5.	10.	15.	30.	45.	60.
250	100	50	100	500	1 : 12,500	+	+	−	−	−	−	−
250	75	50	125	500	1 : 16,666	+	+	−	−	−	−	−
250	50	50	150	500	1 : 25,000	+	−	−	−	−	−	−
250	25	50	175	500	1 : 50,000	+	−	−	−	−	−	−
250	...	50	200	500	...	−	−	−	−	−	−	−

The Protective Action of Blood and Serum.

Blood and serum proteins absorbing ultra-violet rays of wave-length shorter that 3300 Å.U. considerably weaken the action and penetration of the rays of the mercury vapour lamp. From the study of the hæmolytic action of ultra-violet rays on a 2% suspension of washed blood-corpuscles in saline, the protective action of serum can be demonstrated in concentrations up to 1/100. This

technique of the rotary flask which has been employed allowed a very thin film of blood to be exposed to the source of light. Even by this method the biological action of ultra-violet rays penetrating the quartz flask and blood film was weakened roughly 30–40 times. Many of the toxins and antitoxins contain a high percentage of protein substances, and this may explain the reason for their greater resistance to radiation. Cobra venom, however, has a very low protein content, and in the dilution which has been used (*i.e.* 1/2000), this could not be of any significant protective desensitizing power. The addition of defibrinated blood or serum to cobra venom showed that ultra-violet radiation of venom in the presence of a high concentration of protein weakened the toxic action when tested *in vivo* on rabbits and rats.

Experiment No. 5.—The Protective Action of Blood and Serum Protein. Exposure of a Mixture of Cobra Venom Solution in Defibrinated Rabbit's Blood to the Quartz Mercury Vapour Lamp (5720–2300 Å.U.).

30 c.c. of defibrinated rabbit's blood were mixed with 8 c.c. of cobra venom 1/250. 15 c.c. of this mixture was placed in a sterile test-tube and kept in the dark, and the remainder was poured into the sterile quartz flask and irradiated as in Experiment No. 1. Samples of 5 c.c. of this mixture, containing a concentration of cobra venom of 1/1187, were injected into the ear-vein of rabbits. The toxic action of venom and blood mixture was considerably weakened after 30 minutes' irradiation. The post-mortem examination of the rabbits injected with "irradiated venom-blood mixture" showed no sign of intra-vascular clotting (Table V).

TABLE V.

Rabbit No.	Weight in gm.	Dose of venom and blood.	Irradiation; infra-red–2300 Å.U.	Dose of venom per kgm. weight.	Lethal time.	Result.
252	1800	5 c.c.	...	0·00022	54 minutes	Died.
253	1660	5 ,,	15 minutes	0·000254	56 ,,	,,
254	1730	5 ,,	30 ,,	0·00024	148 ,,	,,
255	1670	5 ,,	60 ,,	0·00025	173 ,,	,,

The examination of the centrifuged blood (after the mixture of cobra venom and defibrinated blood had been incubated for 1 hour at 37° C.) showed that the serum was toxic, but the washed corpuscles had no harmful effect.

Irradiation of a Mixture of Cobra Venom and Serum.

By irradiation of a mixture of cobra venom 10 c.c. (1/2500) and horse-serum 10 c.c., the toxic action of venom was weakened, but not destroyed (Table VI)

TABLE VI.

Rat No.	Dose of venom 1/2500.	Horse-serum.	Irradiated.	Lethal time.	Result.
1. 125	2 c.c.	2 c.c.	...	37 minutes	Died.
1. 150	2 ,,	...	...	32 ,,	,,
1. 130	2 ,,	2 c.c.	60 minutes	72 ,,	,,
2. 130	1 ,,	1 ,,	...	36 ,,	,,
2. 160	1 ,,	...	...	40 ,,	,,
2. 120	1 ,,	1 c.c.	60 minutes	120 ,,	,,

Experiment No. 6.—Sensitization of Cobra Venom with Eosin and Irradiation with Visible Rays (5700–3300 *Å.U.*).

Noguchi (1906) has previously shown that a solution of cobra venom in saline sensitized with eosin or erythrosin was destroyed when irradiated with visible rays passing through a glass screen. Further experiments have confirmed those carried out by Noguchi. 6 c.c.'s of a solution of cobra venom 1/2500 concentration in saline was mixed with 1 c.c. of a solution of eosin in saline 1/400. Each 1 c.c. of this solution therefore contained 1/2916 cobra venom and 1/2800 concentration of eosin. Using the same source of light as in all previous experiments, in the presence of eosin the toxic action of venom was destroyed in 15 minutes by the irradiation filtered through window-glass 3300 Å.U. (Table VII).

TABLE VII.

Rabbit No.	Weight in gm.	Cobra venom 1/2916 and eosin 1/2800.	Dose of venom per kgm. weight.	Irradiation; infra-red–3300 Å.U.	Lethal time.	Result.
246	2070	1 c.c.	0·00017	...	80 minutes	Died.
248	2420	1 ,,	0·00014	15 minutes	...	Alive.
249	1620	1 ,,	0·00021	30 ,,	...	,,
250	2070	1 ,,	0·00017	60 ,,	...	,,

Experiment No. 7.—Sensitization of Cobra Venom with Eosin in the Presence of Blood and Serum.

A mixture of cobra venom and a solution of eosin with defibrinated rabbit's blood, rabbit's serum or horse-serum exposed to visible rays as in Experiment No. 6 was unaffected by prolonged irradiation.

30 c.c. of rabbit's defibrinated blood, 2 c.c. of eosin in saline 1/100 and 10 c.c. of cobra venom 1/2500 were prepared. Each 5 c.c. of this mixture containing 3·57 c.c. of defibrinated blood, 1·19 c.c. of cobra venom 1/2500, 0·24 c.c. of eosin 1/100 were injected intravenously into rabbits. After 60 minutes' irradiation there was evidence of destruction of the hæmolysin and cytolysin of cobra venom, but the toxic effect was still maintained. Similar results were

obtained when rabbit's serum and horse-serum were used in place of rabbit's defibrinated blood. The results of these experiments agree with the other results which have been obtained with photo-dynamic sensitizers and blood-corpuscles in the presence of serum and proteins.

Experiment No. 8.—The Effect of Irradiation on Antivenin.

Antivenin (Burroughs, Wellcome) irradiated in a quartz flask by the same method as in all previous experiments was found to be resistant to ultra-violet irradiation. The protective action against a lethal dose of venin appeared to be identical with normal and irradiated antivenin, when tested in *vivo*, or *in vitro* by examination of the hæmolytic action on washed blood-corpuscles. It was also found possible to destroy venin in the presence of antivenin by irradiation of this mixture. An excess of venin mixed with antivenin was prepared which was lethal to rabbits when injected by the intravenous route; this was rendered non-toxic after irradiation with the mercury vapour lamp. Further experiments which were carried out indicated that sensitization of antivenin with eosin and intensive irradiation did not destroy its positive action against the lethal action of cobra venom. All these experiments have proved that antivenin is very resistant to the action of light.

DISCUSSION.

The spectrophotographic examination of a 1/500 solution of cobra venom in saline shows that there is absorption of ultra-violet rays shorter than 2540 Å.U., while the bands from 2570 Å.U. are weakened. This is very different to the spectrophotographic examination of the typical protein solutions, which usually demonstrate complete absorption from 3700 Å.U. The characteristic spectrum may explain the reason why the neurotoxin of cobra venom is only destroyed by wave-lengths shorter than 2800 Å.U. this being the region of absorption. The experiments which have been described definitely prove that the hæmolysins and cytolysins can be destroyed by longer wave-lengths, *i.e.* irradiation through vita-glass (3300–2800 Å.U.). These results confirm other experiments, which demonstrated that the kinase of oxalate blood and tissue extracts could be destroyed by irradiation; injection of such irradiated blood or extracts rendered blood incoagulable and prevented intravascular clotting. It is interesting to observe that the effects of heat and ultra-violet light appear to be contrary to each other: cobra venom is resistant to heat at 80°–100° C., and is readily destroyed by irradiation; antivenin is sensitive to heat but resistant to the action of light. The spectrophotographic examination of antivenin showed complete absorption of all rays shorter than 3700 Å.U.; the spectrophotograph is similar to that of a solution of horse-serum. Antivenin is prepared as a serum, and the resistance to irradiation could be explained by the absorption of the biologically active ultra-violet rays by the serum. But even on this assumption it would be difficult to explain the destruction of venom which takes place in the presence of serum or antivenin. The results of these experiments indicate that the active principles of venom and antivenin show different behaviours to irradiation, and this in all

probability is due to the nature of their chemical structures. It also suggests that this technique of irradiation could be employed in the destruction of toxin, in a mixture of toxin and antitoxin.

SUMMARY.

As a result of irradiation of cobra venom and antivenin it has been shown that—

1. The neurotoxin present in a solution of cobra venom in saline was destroyed only by rays shorter than 2800 Å.U. unless sensitized with eosin.
2. The photodynamic action of eosin is arrested by the presence of blood and serum.
3. Hæmolysins and cytolysins were destroyed by rays longer than 2800 Å.U., but not by rays longer than 3300 Å.U.
4. Antivenin is resistant to prolonged irradiation as obtained in serum.

REFERENCES.

NOGUCHI, H.—(1906) *J. Exp. Med.*, **8**, 252.

THE ANAPHYLACTIC REACTION OF THE ISOLATED UTERUS OF THE RAT.

C. H. KELLAWAY.

From the Walter and Eliza Hall Institute, Melbourne.

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THE striking similarity of the symptoms produced in the dog and guinea-pig in anaphylactic shock to those which follow the injection of peptone (Biedl and Kraus, 1910) and histamine (Dale and Laidlaw, 1910), early directed attention to the possibility that the liberation of histamine might be the ultimate step in the anaphylactic reaction. The experiments of Lewis and his colleagues (1927) on the local anaphylactic response of sensitive skin have demonstrated that these reactions, like the "triple response" to injury, are mediated by the liberation of a histamine-like substance. Finally, Best, Dale, Dudley and Thorpe (1927) have shown that histamine is a normal constituent of the cells of mammalian tissues. Dale (1929), reviewing the evidence in his recent Croonian lectures, concludes that histamine, if not identical with Lewis's H-substance, is at least its active constituent.

One of the anomalous physiological actions of histamine is upon the uterus of the rat, which, as Guggenheim (1912), Longcope (1922) and Voegtlin and

Dyer (1924) have shown, responds by relaxation instead of contraction. It is therefore of some interest to ascertain whether the anaphylactic reaction of the rat's plain muscle is one of contraction or of relaxation.

Unfortunately the rat is extremely difficult to sensitize, usually responds to a "shocking" intravenous injection with only slight symptoms and exhibits very marked individual variation. In these respects its behaviour is opposite to that of the guinea-pig (Doerr, 1929). Apart from local anaphylaxis, as demonstrated by Arthus (1903) and Gerlach (1925), a whole series of investigations have failed to produce definite evidence of anaphylaxis in this animal. Rosenau and Anderson (1906), Tromsdorff (1909), Uhlenhuth, Haendel and Steffenhagen (1910), Novy and de Kruif (1917), Longcope (1922) and Ebert (1927) all obtained negative results. Parker and Parker (1924) were, however, successful in producing both active and passive sensitization in the white rat to the sera of the sheep and horse. Their results exhibit extreme individual variation, and there were only four deaths in a large number of experiments. Using very young rats (50 to 75 gm. in weight) passively sensitized to horse-serum, they obtained (among a large number of negative results) contraction of the uterus with horse-serum in concentrations of 1 in 100 and 1 in 200 in two experiments only. They showed that the plain muscle of the normal rat was not affected by horse-serum in a somewhat greater concentration, but did not demonstrate desensitization following contraction, and produced no evidence whatever that the contraction which they observed was anaphylactic in nature.

EXPERIMENTS ON ACTIVE SENSITIZATION.

The protein used for sensitization and testing was crystalline hen egg albumin, samples of which, several times crystalized, were prepared for me by Mr. H. F. Holden and Miss Mavis Freeman.

The rats were a mixed stock, which originally were obtained from the Imperial Cancer Research Laboratories, London, some black, some albino, and some black and white. Fairly young rats were used for sensitization, which was attempted by repeated subcutaneous injection of 10 or 40 mgm. at 3- to 4-day intervals, and in one small series by a single subcutaneous injection of 50 mgm. of protein. Fifteen rats were tested by Dale's method from 8 to 25 days after the final injection. The bath used was of 100 c.c. capacity. A smaller one of 50 c.c. was found to be unsatisfactory, as the hair by which the uterus was suspended tended to stick to the side of the bath when frothing occurred after the addition of protein. In only two experiments were positive results obtained. One of these was upon a rat which received 14 subcutaneous injections of 10 mgm. of egg albumin. On the 20th day after the last injection 20 mgm. of egg albumin were administered intravenously. The injection was followed by collapse like that described by Parker and Parker. Recovery occurred in about 10 minutes. After an interval of 10 weeks a single subcutaneous injection of 10 mgm. was given in the hope of restoring sensitiveness. The uterus was tested on the 10th day after this injection when the rat weighed 129 gm., and gave a definite contraction with a dose of 20 mgm. with one horn and of 50 mgm. with the other. A second dose in each case after changing the Ringer elicited no response. Three other rats similarly treated and tested

on the 8th, 10th and 11th days gave negative results. The other rat which gave a definite result was one of 6 in which sensitization was attempted by 6 successive subcutaneous injections each of 40 mgm. of egg albumin. The isolated uteri of these rats failed to react to test doses of 40 to 50 mgm. of egg albumin on the 8th, 14th, 17th and 25th days when the rats weighed 140 to 150 gm., except that of one animal tested on the 25th day. One horn of the uterus of this rat contracted on the addition of 40 mgm. of egg albumin (Fig. 1) to the bath and the other horn gave no contraction with 10 mgm. The first horn after changing the Ringer failed to react with a second dose of 40 mgm.

Three rats weighing 110, 105 and 101 gm. tested on the 13th, 18th and 20th days after 14 subcutaneous injections each of 10 mgm. gave negative results, as also did two rats tested on the 12th day after a single subcutaneous injection of 50 mgm. of egg albumin.

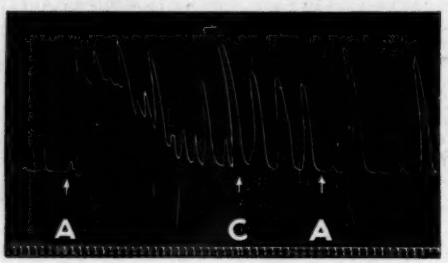

FIG. 1.—Responses of one horn of the isolated uterus of a rat weighing 150 gm. which had received 6 subcutaneous injections each of 40 mgm. of egg albumin at intervals of 3 or 4 days. The uterus was tested 25 days after the last injection, and the second horn failed to react with a dose of 10 mgm. of egg albumin. At A, 40 mgm. of egg albumin were added to the bath containing 100 c.c. of Ringer's solution. At C the Ringer's solution was changed. Time in ½-minute intervals.

The doses used for testing the isolated uteri ranged from 10 to 100 mgm. In numerous experiments with normal rats the uterus was never observed to contract with egg albumin in these concentrations (1 in 10,000 to 1 in 1000). The two positive results in actively sensitized rats were obtained with concentrations of 1 in 5000 and 1 in 2500. The results in these two experiments only acquire significance in view of the regularity with which contraction can be obtained with the uteri of rats passively sensitized by the intra-peritoneal injection of rabbit anti-egg albumin serum.

Using freshly made-up solutions of egg albumin I have not observed any signs of toxic effects from the injection even of large doses in normal rats similar to those mentioned by Parker and Parker. Possibly this was because I used young animals. The egg albumin was weighed out and dissolved up in normal physiological saline and used at once for testing or sensitizing injections. On one occasion this rule was broken. A series of 12 rats weighing about 135 gm. which had received two subcutaneous injections, the first of 10 and the second of 20 mgm. of egg albumin at 5 days' interval, received after an

interval of 8 days a third dose of 20 mgm. subcutaneously. All the animals collapsed and died within 6 hours. The solution of egg albumin used for this injection, though sterile, had been kept for nearly a fortnight at 0° C. and its lethal effects were possibly due to the presence of toxic decomposition products.

EXPERIMENTS UPON PASSIVE SENSITIZATION.

In contrast to the irregularity of the results of active sensitization, passive sensitization as judged by the reaction of the isolated smooth muscle is an extremely regular phenomenon. Sensitiveness is acquired in approximately the same time as in the guinea-pig, and depends upon the dose of serum used for sensitization. Intravenous injection of egg albumin even in large doses into passively sensitized animals only occasionally produces the typical symptoms described by Parker and Parker (1924), though the plain muscle of

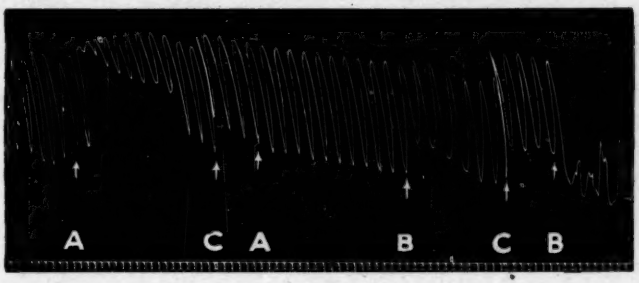

FIG. 2.—Responses of one horn of the isolated uterus of a rat passively sensitized to egg albumin by the intra-peritoneal injection 48 hours before testing of 1·0 c.c. of rabbit anti-egg albumin serum. At A, 2·0 mgm. of egg albumin, and at B, 0·4 mgm. of histamine were added to the bath. At C the Ringer's solution was changed. Time in ½-minute intervals.

animals similarly sensitized gives good responses with concentrations as small as 1 in 100,000 and 1 in 50,000 (1·0 to 2·0 mgm. in a bath of 100 c.c.).

The results in a small typical series of animals are set out in Table I. The rabbit serum used for sensitization was prepared for me by Miss F. E. Williams. Three rabbits received intravenously 1 c.c. of a 20% solution of crystalline egg albumin and four days later 2·0 c.c. of the same solution. They received five further injections of 2·0 c.c. at 3 to 4 days' interval. When bled out 4 days after the last injection the pooled serum tested against 1% egg-albumin solution gave good precipitation in dilutions from 1 in 2 to 1 in 512.

In all cases the response of the uterus was a contraction (Fig. 2), and a second dose of egg albumin after changing the Ringer's solution caused no response.

A number of rats were sensitized by intravenous injection of immune serum and it was found that they were most sensitive after 24 to 36 hours. Intravenous sensitizing injections did not yield so high a degree of sensitiveness when the animals were tested after 48 hours as that resulting from intraperitoneal injection.

Despite the regularity of the response of the isolated plain muscle the

production of symptoms in these passively sensitized animals by intravenous injection of egg albumin was an infrequent event.

Rats weighing from 105 to 130 gm. were sensitized passively by the intraperitoneal injection of 1·0 c.c. of the rabbit antiserum, and received varying doses of crystalline egg albumin intravenously into a lateral vein of the tail. Two received 200 mgm., two 50 mgm., one 40 mgm. and one 20 mgm. All were tested 48 hours after the sensitizing injection and one only showed any symptoms following the injection. The rat which received 20 mgm. of egg albumin in 0·4 c.c. of saline was obviously sick within 3 minutes, and in 4

TABLE I.—*Results of Tests of Isolated Uteri of Rats Passively Sensitized by Intraperitoneal Injection of Rabbit Immune Serum and Tested 42 to 50 Hours Later.*

Weight of rat in gms.	Dose of immune serum in c.c.'s.	Dose of egg albumin in 100 c.c. bath, in mgms.	Result.	
135	0·5	2	Right horn	Very small response.
		5	Left horn	Small response.
105	0·5	10	,,	Good response.
122	0·5	25	Right horn	Moderate response.
		50	Left horn	Good response.
123	0·5	10	,,	,,
136	1·0	5	,,	,,
150	1·0	2	,,	Moderate response.
130	1·0	2	Right horn	Good response.
		1	Left horn	Moderate response.
130	1·0	2	,,	Good response.
150	1·0	2	,,	,,
163	1·0	2	,,	,,

minutes after the injection became collapsed. The animal remained in a profoundly collapsed condition for 8 minutes, and then rapidly improved, and was completely recovered 3 hours after the injection.

The injection of egg albumin intravenously caused desensitization of the plain muscle whether symptoms were produced by the injection or no. The uterus of one of the surviving rats which had no symptoms following the injection of 40 mgm. of egg albumin and also that of the rat which had moderate symptoms after the injection of 20 mgm. were tested 4 hours after the injection. They gave no reaction with a dose of 100 mgm. of egg albumin in a bath of 100 c.c.

In order to determine finally whether the contraction observed in the passively sensitive rat uterus following the addition of egg albumin to the bath was truly anaphylactic, I performed the experiment of protecting the uterus *in vitro* from desensitization by the anaphylactic antigen, by the presence of excess of immune serum in the bath, as Dale and Kellaway (1921) had demonstrated could be done with guinea-pig plain muscle. The experiment is much more difficult in the rat than in the guinea-pig, because much larger quantities

of precipitating antibody must be present in the bath to protect the plain muscle from the dose necessary for testing, which is 20 times as great as the dose used in the experiment with the guinea-pig plain muscle.

Two types of experiment were performed. In one after ascertaining on one horn of a sensitized rat that the plain muscle gave a good contraction with 2 mgm. of egg albumin, the other horn was put up in the bath, and rabbit anti-egg albumin serum was added in successive doses of 0·5 c.c. until 6 c.c. of serum had been added (Fig. 3). 2·0 mgm. of egg albumin in 0·2 c.c. was now added to the bath and the uterus maintained its normal rhythm. The Ringer's solution in the bath was now changed, and normal rabbit serum was added in twelve successive amounts of 0·5 c.c. On adding 2·0 mgm. of egg albumin to the bath the uterus gave a definite specific contraction and thereafter was desensitized, as was shown by changing the Ringer's solution and giving a third dose of 2·0 mgm. of egg albumin, which produced no effect. For this experiment, sera which were more than a fortnight old were used, so as to avoid the non-specific stimulant action of serum on the plain muscle, and

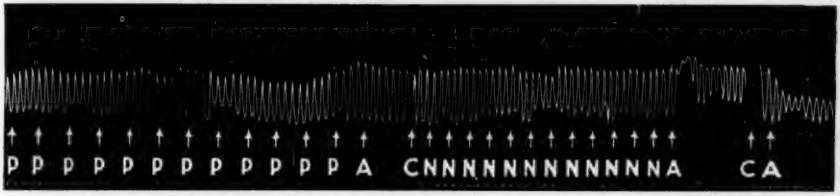

FIG. 3. – Responses of one horn of the isolated uterus of a rat passively sensitized to egg albumin by the intraperitoneal injection 46 hours before testing of 1·0 c.c. of rabbit anti-egg albumin serum. At P, 0·5 c.c. of rabbit anti-egg albumin serum. at A, 2·0 mgm. of egg albumin, at N, 0·5 c.c. of normal serum added to the bath. At C the Ringer's solution was changed. Time in 1-minute intervals.

sufficient time was allowed between each addition of serum so as to permit of a gradual rise in the concentration of serum in the bath.

The other type of experiment, of which a number were carried out, was not so satisfactory though the result was invariably clear. Mr. H. F. Holden prepared for me solutions of the globulins from rabbit anti-egg albumin precipitating serum and from normal rabbit serum. The globulin solution equivalent to 12 c.c. of normal rabbit serum invariably failed to prevent egg albumin in a dose of 2·0 mgm. from causing its specific contraction, though the result was complicated by the difficulty of keeping the uterus sufficiently relaxed during the addition of such large amounts of protein. When the Ringer's solution was changed a second dose of egg albumin invariably failed to cause any contraction (Fig. 4).

The result in the case of globulins from rabbit anti-egg albumin serum was opposite to this. Globulins equivalent to 8 or 10 c.c. of serum invariably protected the uterus from 2 mgm. of egg albumin, but after changing the Ringer's solution in the bath a second dose gave a large contraction. After again changing the Ringer's solution a third dose caused no response (Fig. 5). When globulin solution equivalent to 4·0 c.c. of rabbit anti-egg albumin serum

was added to the bath it failed to protect the sensitive uterus from a dose of 10 mgm. of egg albumin, and a second similar dose of egg albumin after changing the Ringer's solution caused no response. This last experiment demonstrates the quantitative nature of the protection observed in these experiments.

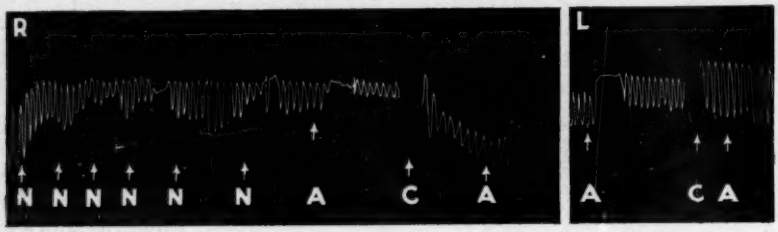

FIG. 4.—Responses of right (R) and left (L) horns of the isolated uterus of a rat passively sensitized to egg albumin by the intraperitoneal injection 44 hours before testing of 1·0 c.c. of rabbit anti-egg albumin serum. At N, globulins equivalent to 2 c.c. of normal rabbit serum, at A, 2·0 mgm. of egg albumin added to the bath. At C the Ringer's solution was changed. Time in 1-minute intervals.

The contraction regularly caused in the plain muscle of rats prepared 48 hours earlier by the intraperitoneal injection of rabbit immune serum is a genuine anaphylactic response, for (1) the reaction desensitizes the plain muscle

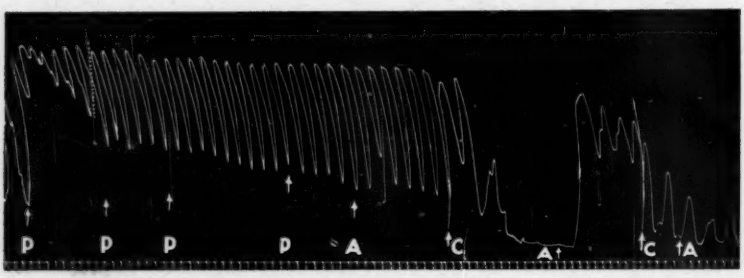

FIG. 5.—Responses of one horn of the isolated uterus of a rat killed 48 hours after the intraperitoneal injection of 1·0 c.c. of rabbit anti-egg albumin serum. At P, globulin solution equivalent to 2·0 c.c. of rabbit anti-egg albumin serum. At A, 2·0 mgm. of egg albumin added to the bath. At C the Ringer's solution was changed. Time in ½-minute intervals.

so that it fails to respond to a second dose on washing out the bath, (2) the plain muscle can be desensitized *in vivo* by intravenous or subcutaneous injection of egg albumin some hours before testing the plain muscle, and (3) the plain muscle can be protected *in vitro* both from reaction and from desensitization *if sufficient* rabbit anti-egg albumin antibodies are present before egg albumin is added to the bath.

DISCUSSION.

It is evident that the anaphylactic reaction of the isolated sensitive plain muscle of the rat is one of contraction and not of relaxation, and that this reaction is due to the interaction of (heterologous) precipitating antibody, fixed in the plain muscle-cells, with the specific anaphylactic antigen. It also appears to be extremely unlikely that histamine is responsible for this anaphylactic manifestation. In the first place the rat is very insensitive to histamine. According to Voegtlin and Dyer (1924) the intravenous lethal dose for the white rat is 300 mgm. of the base per kilo, 1 in 3,000,000 causes definite relaxation and 1 in 300,000 abolition of the rhythm of isolated plain muscle. In my experiments relaxation was obtained with concentrations of 1 in 1,000,000 to 1 in 100,000 of the base and the rats used tolerated large doses of histamine intravenously without any symptoms. In view of the insensitiveness of the rat to histamine it is unlikely that the production of histamine plays any part in the production of anaphylactic symptoms in the whole animal. It might, indeed, be argued that the insensitiveness of this species to histamine actually determines the difficulty and irregularity with which anaphylactic phenomena are produced in the intact animal either actively or passively sensitized, and that in the rat when symptoms appear these are due to the production of histamine. But the anaphylactic contraction of the passively sensitized plain muscle is a regular phenomenon, and unless it were shown either that the sensitized uterus, unlike the normal uterus of the rat, responds to histamine by contraction, or that the sensitized uterus in the presence of foreign protein reacts in this way, it is evident that histamine can play no part in causing the anaphylactic reaction of the isolated smooth muscle.

The passively sensitive uterus of the rat before being desensitized behaves to histamine exactly like the uterus of the normal rat, relaxing with the same concentrations of histamine, but normal rat muscle in the presence of egg albumin is less sensitive to histamine, and a higher dosage is needed to cause relaxation or abolition of rhythm if protein and histamine are added together, or if histamine is added after the addition of protein to the bath. A sensitized uterus which has just contracted in response to the specific anaphylactic antigen, and is insensitive to further doses of antigen, behaves like a normal uterus with histamine if the Ringer's solution be changed before histamine is added to the bath, but if histamine be added before changing the Ringer's solution, a dose which normally causes relaxation fails to do so, and in two experiments a small contraction was actually observed (Fig. 2). This phenomenon is so irregular as to be of doubtful significance.

Taking all the evidence together it appears almost certain that histamine plays no significant part in the production of anaphylactic phenomena in the rat.

The results recorded here are also of interest in another connection. The work of Doerr and Russ (1909), Weil (1916) and others has led to the general acceptance of the view that precipitin and anaphylactic antibody are identical. Longcope (1922) has shown that in the white rat precipitating antibody begins to appear on the 3rd or 4th day following the last of repeated intraperitoneal injections of horse-serum, and regularly reaches its maximal

intensity on the 6th to 8th day. He was unable to confer passive sensitization on guinea-pigs with such sera. Here, then, is one reason for the difficulty of inducing anaphylactic sensitiveness in the rat. The body-cells in the rat do not readily fix the homologous precipitating antibody, even though, as I have shown, they regularly fix heterologous (rabbit) precipitating antibody. In view of the occasional occurrence of active sensitization of the rat plain muscle, it is perhaps doubtful if we should accept Longcope's conclusion that in the white rats anaphylactin and precipitin are not identical.

The difficulty with which homologous precipitating antibody is fixed to the tissue-cells is not the whole explanation of the irregularity of anaphylactic phenomena in this species. Despite the regularity with which passive sensitization of uterine plain muscle may be demonstrated, the passively sensitive rats are still only irregularly sensitive, as judged by the production of symptoms following intravenous injection of antigen. The further explanation which must be invoked is the general resistance which the rat exhibits to poisons of all kinds (*cf.* Voegtlin and Dyer). If a toxic substance be produced by the union of anaphylactic antibody and specific anaphylactic antigen in the cells of the body, it must as a rule be produced in insufficient amount to cause symptoms.

Finally, it is clear that the contraction of plain muscle demonstrated in passively sensitized rats cannot play any significant part in the production of general anaphylactic symptoms when these occur.

It is a pleasure to acknowledge my indebtedness to Mr. H. F. Holden, Miss Mavis Freeman and Miss F. E. Williams for their help in these experiments.

REFERENCES.

Arthus, M.—(1903) *C.R. Soc. Biol.*, **55**, 819.
Best, C. H., Dale, H. H., Dudley, H. W., and Thorpe, W. V.—(1927) *J. Physiol.*, **62**, 397.
Biedl, A., and Kraus, R.—(1910) *Zbl. Physiol.*, **24**, 258.
Dale, H. H.—(1929) *Lancet*, **1**, 1233, 1285.
Dale, H. H., and Kellaway, C. H.—(1921) *J. Physiol.*, **54**, p. cxliii.—(1923) *Phil. Trans. Roy. Soc.*, B, **211**, 273.
Dale, H. H., and Laidlaw, P. P.—(1910) *J. Physiol.*, **41**, 344.
Doerr, R.—(1929) Kolle & Wassermann's 'Handbuch der pathogenen Mikroorganismen,' 3rd ed., **1**, 759.
Idem and Russ, V. K.—(1909) *Z. ImmunForsch.*, **3**, 181, 706.
Ebert, M. K.—(1926) *Ibid.*, **51**, 79.
Gerlach, W.—(1925) *Verhand. deuts. path. Ges.*, **20**, 272.
Guggenheim, M.—(1912) *Ther. Mschr.*, **26**, 795.
Lewis, T.—(1927) 'The Blood Vessels of the Human Skin and their Responses.' London (Shaw & Sons).
Longcope, W. T.—(1922) *J. Exp. Med.*, **36**, 627.
Novy, F. G., and de Kruif, P. H.—(1917) *J. Infect. Dis.*, **20**, 776.
Parker, J. T., and Parker, F.—(1924) *J. Med. Res.*, **44**, 263.
Rosenau, M. J., and Anderson, J. F.—(1906) *Bull. U.S. Hyg. Lab.*, No. 29, p. 57.
Tromsdorff, R.—(1909) *Arb. Gesundhmt.*, **32**, 560.
Uhlenhuth, Haendel and Steffenhagen.—*Zbl. Bakt.*, 1 Abt. Ref., **67** (Suppl.), 158.
Voegtlin, C., and Dyer, H. A.—(1924) *J. Pharmac. Exp. Ther.*, **24**, 101.
Weil, R.—(1916) *J. Immunol.*, **1**, 19, 35, 47.

CAROTENE AND VITAMIN A : THE ANTI-INFECTIVE ACTION OF CAROTENE.

H. N. GREEN AND E. MELLANBY.

From the Department of Pharmacology, University of Sheffield.

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RATS fed on a diet devoid of vitamin A but complete, so far as is known, in other dietetic units ultimately develop multiple infective lesions and die (Green and Mellanby, 1928). We have previously dealt with this phenomenon, and also attempted to see whether the experimental observation in animals had any clinical significance by giving large amounts of vitamin A for therapeutic purposes to a small number of patients suffering from puerperal septicæmia (Mellanby and Green, 1929). In this report we wish to deal with a specific problem, namely, as to whether carotene has the characteristic anti-infective action previously shown to be possessed by vitamin A.

The prolonged discussion on the relation between the carotenoid pigments and vitamin A has now been placed on a more definite footing by the work of Euler, Euler and Hellström (1928), who have shown that samples of crystalline carotene, presumably pure, possess growth-promoting properties similar to those of vitamin A. Many other carotenoid pigments were tested, but only one, α-dihydrocrocetin, was found to be active (Euler, Euler and Karrer, 1929).

The literature of the sequence of events which at one time and another has led to a denial of this relationship is surveyed in two recent papers—Collinson, Hume, Smedley-Maclean and Smith (1929), and Moore (1929)—and therefore need not be further discussed here. The present position, however, is still a little obscure since, on the one hand, Dulière, Morton and Drummond (1929), using a sample of carotene of a high degree of purity, found it inactive. At present it is suggested that their use of a fat-free basal diet may explain the discrepancy, for it is possible that carotene is only absorbed in the presence of fat. On the other hand, the work of Collinson *et al.* (1929) and Moore (1929) indicates that increasingly pure samples of carotene do not possess, at any rate, less " vitamin A " activity.

It is clear that, if carotene has the properties usually associated with vitamin A, and even if carotene is the actual compound responsible for the activity of substances such as green and other vegetables, butter and egg yolk, as it might well be, it is not the same substance as vitamin A in liver fats. The carotene content of cod or mammalian liver fat can only at the best be very small, yet preparations from these substances can be obtained which have as much or even more vitamin A activity than crystalline carotene. Liver oils containing vitamin A give with $SbCl_3$ a transient blue colour with an absorption

band at 610–630 $\mu\mu$, whilst carotene gives a permanent blue colour with the same reagent with an absorption band at 590 $\mu\mu$. Again, vitamin A in liver oils can be readily extracted from the unsaponifiable fraction by alcohol, whilst carotene is only slightly soluble in alcohol. It is possible that the association of vitamin A with other lipoid material when prepared from liver oils may modify some of its attributes, but the weight of evidence is strongly in favour of its being a substance other than carotene.

Most of the experimental work on carotene and its relation to vitamin A has depended on observations concerning its effect on the weight of rats which have begun to lose weight as the result of vitamin A deprival. Our observations on the action of vitamin A in raising the resistance of the body to auto-infection seemed so important that we felt it desirable to test the action of carotene in its relation to infection. Obviously if carotene has the biological properties of vitamin A it should be equally effective in preventing the occurrence of this generalized infection. Preliminary experiments quickly indicated that it was probably as efficient as vitamin A in this respect, and further work was only required to determine the minimal doses which had complete protective and curative effects.

METHODS AND RESULTS.

The feeding technique was in all essentials that previously described by us (1928). The basal diet devoid only of vitamin A contains a considerable quantity of fat chiefly in the form of heated olive oil, and the calcium-phosphorus ratio is a good one.

The animals used were albino rats bred from stock rats which have been reared under standard conditions for several years.

Vitamin D was given in every case, with the exception noted in the tables, each rat receiving $\frac{1}{2}$ drop of radiostol (irradiated ergosterol) daily.

The sample of crystalline carotene used had a melting-point of 174° (in air), and the same specimen was used throughout. It was dissolved in freshly distilled ether. The required volume of solution was allowed to evaporate on a little casein, which was then intimately mixed with a little of the basal diet. The "bit" so made was consumed by the rat before any more food was given. Control rats received an equivalent amount of extra casein. The ethereal solutions of carotene were prepared weekly and stored in the refrigerator.

The experimental findings as set out in tabular form are in the main self-explanatory. It will be noticed that in Experiment I there was no preliminary depletion period on a —A diet; that is to say, the experiment was started at a time when there was still a supply of vitamin A in the livers and other organs of the animals. Some of these animals were fed on diets containing varying quantities of carotene and others were used as controls and given the —A diet. In Experiments II and III, a preliminary depletion period on a —A diet of 32 and 52 days' duration respectively was introduced. During these preliminary periods the animals were thus deprived of their vitamin A stores, and many of them in Experiment III developed infective foci and lost weight. In this experiment the supplementary carotene had therefore often to bring about partial or complete recovery from the infected state before resumption

of growth and improvement in health could be effected. Thus in Experiment I the test consisted essentially of determining the prophylactic, and in Experiment III the curative action of carotene.

DISCUSSION OF RESULTS.

It is obvious from the above results that carotene acts as a very potent anti-infective agent. From Tables I–IV it will be seen that the response to carotene is as reasonably quantitative as could be expected. Complete protection against infection was always found when greater amounts than 0·04 mgm. of carotene were given daily, even when a prolonged depletion period on a — vitamin A diet had preceded the addition of the carotene. Many of these animals must have developed an infection in the depletion period (on the basis of previous results) and yet complete recovery and good health followed. In the case of one animal in Experiment III receiving 0·04 mgm. of carotene no recovery followed and the animal died on the 107th day. This is an exceptional result, for even when only 0·02 mgm. of carotene was given to 3 animals in the same experiment they remained healthy and continued to grow throughout the feeding period. In the case of the animals receiving only 0·01 mgm. of carotene the protection against infection was less certain, and some died with organs invaded by micro-organisms. With 0·005 mgm. of carotene the protection afforded was still less, although even in this case, when the animals are compared with the controls receiving no carotene, there is evidence of an increased resistance. It is difficult to state the exact protective dose of carotene in the case of the rat, but probably in the average animal when no running-out period (*i. e.* deprival of vitamin A) is allowed, a daily dose of 0·02 mgm. is sufficient. After the animal's body has been completely deprived of vitamin A and infective foci have probably started prior to the addition of carotene, a dose of 0·02 mgm. seems to be enough to restore perfect health and

TABLE I.—*The Comparative Effects of Varying Quantities of Carotene on Resistance to Infection. Exp. I: No Preliminary Depletion Period.*

Duration of experiment, 119 days.

Nature of supplement.	Survival time.	Site and intensity of infection.	Growth response (in gm.).	Remarks.
0·01 mgm. carotene	To end of experiment	M.E.	35–122	General condition good.
Ditto	Ditto	M.E.; mastoid	34–140–131	„ „
„	„	M.E.	40–130	„ „
0·005 mgm. carotene	„	T.A.++	35–119–97	Scanty abdominal fat.
Ditto	„	T.A.++	39–124–111	„ „
„	106	X.+; T.A.+; M.E.+; bladder stone	36-96-76	No „
Nil	50	T.A.+++; enteritis; uterus (cornu); X.+	47–119–106	...
„	39	X.+; T.A.+++; N.S.; bladder; kidney	42–129–121	...
„	59	T.A.+++; M.E.++	39–101–84	...

T.A. = Tongue abscess. X. = Xerophthalmia. N.S. = Nasal sinus. M.E. = Middle ear.

TABLE II.—*Exp. II: Partial Preliminary Depletion Period. (32 Days on —A Basal Diet prior to adding Supplement.)*

Duration of experiment, 125 days.

Nature of supplement after partial depletion period.	Survival time.	Site and intensity of infection.	Growth response (in gm.).	Remarks.
0·005 mgm. carotene	To end of experiment	T.A.+++; colon	115–142–122	Scanty abdominal fat thymus very small.
Ditto	Ditto	X.+; T.A.+++	125–150–136	Ditto.
„	„	T.A.+	106–134–95	Thymus fair size; fair amount abdominal fat.
„	107	T.A.±; mastoid; N.S.; bladder	94–122–86	Bladder hypertrophied, with shaggy inflamed mucous membrane; left ureter greatly dilated; pelvis of left kidney dilated and full of infected necrotic material.
0·01 mgm. carotene	42	...	105–90	Cause of death unknown.
Ditto	To end of experiment	T.A.±	120–162	Normal thymus; fair amount abdominal fat.
„	70	X.+; M.E.++	110–100	...
„	64	Enteritis	85–90–75	...
0·01 mgm. carotene (No vitamin D)	77	Cæcum and colon; bladder	97–124–86	Litter 14 days previous to death; hæmaturia prior to death.
Ditto	To end of experiment	...	108–140	Normal thymus; fair amount abdominal fat.
„	Ditto	...	105–139–133	Healthy.
„	„	...	96–148	„
0·02 mgm. carotene (*3 rats*)	„	...	97–143 (average)	All healthy.
0·04 mgm. carotene	107	M.E.+++; N.S.	113–83	No recovery (exceptional result).
Ditto (*3 rats*)	To end of experiment	...	107–156 (average)	All healthy.
0·08 mgm. carotene (*4 rats*)	Ditto	...	114–170 (average)	„
0·05 gm. dried cabbage (*4 rats*)	„	...	78–171 (average)	All healthy; more abdominal fat than 0·08 carotene group.
Nil	70	T.A.+++; enteritis; bladder; seminal vesicles	119–136–94	...
„	80	X.+; T.A.++++; kidneys	120–125–104	Kidneys studded with small septic foci; ureters enormously distended with necrotic septic material; bladder almost unaffected; hyperplasia cardiac end of stomach.
„	54	X.+; T.A.+	128–135–103	...
„	84	X.+++; T.A.+++; bladder	95–105–96	Bladder showed great hyperplasia with red sessile projections from mucous membrane.

growth. When cabbage was used as a source of carotene, 0·5 gm. of the dried leaf, equivalent to about 5 gm. of cabbage, always afforded complete protection. In the case of cod-liver oil—a substance containing no carotene but vitamin A in an unknown form—the protection was not complete although 10 mgm. of this substance was used as the supplement. The brand of cod-liver oil was not a particularly good one as regards its vitamin A content, for, as determined by

TABLE III.—*Exp. III: Preliminary Depletion Period. (52 Days on —A Basal Diet prior to adding Supplement.)*

Duration of experiment (total), 161 days.

Nature of supplement after depletion period.	Survival time.	Site and intensity of infection.	Growth response (in gm.).	Remarks.
0·02 mgm. carotene	To end of experiment	None	111–152	...
„	Ditto	„	106–159	...
„	54	T.A.++; X.+; cystitis	...	Marked hæmaturia prior to supplement; no recovery from depletion period.
0·01 mgm. carotene	62	M.E.++; N.S.; X.++	104–85	...
„	To end of experiment	None	97–124	...
„	Ditto	„	101–136	...
0·005 mgm. carotene	84	T.A.++; pyonephrosis; bladder stone	92–71	...
„	73	Mastoid; enteritis	101–84	Enteritis prior to supplement.
0·01 mgm. carotene + 0·5 gm. dried cabbage (*2 rats*)	To end of experiment	None	99–129 89–111	...
0·5 gm. dried cabbage (*3 rats*)	Ditto	„	101–147 99–123 84–101	...

the tintometer method of Carr and Price (1925), it only contained 4 units as compared with 9 units or more that would be found in a potent specimen.

The relation of the anti-infective action of carotene to growth requires some consideration, for the crucial biological test of vitamin A has always up to the present depended on its power to influence growth rather than its power to confer resistance to bacterial invasion. It must be pointed out that the experimental periods used in our experiments are much longer than those of other workers. It has been usual to observe the effects of carotene on growth over periods of 28 to 35 days, whereas in our experiments observations were made over periods up to 80 days. It will be noticed that on the whole where no signs of infection were found post-mortem the animals continued to grow to the end of the experiment. Occasionally growth continued, although some infection, generally of a mild type, was found post-mortem. Thus some

infection is compatible with growth. This was especially noticeable in the cod-liver oil group, where the infective foci were slight in degree. It would seem probable from this that cessation of growth and loss of weight under these conditions always indicated an infected state. This, although mainly true, we hesitate to say is always the case, for in a few animals no infective foci

TABLE IV.—*Exp. IV: The Comparative Effects of Carotene, Cod-Liver Oil and Cabbage on Resistance to Infection. No Preliminary Depletion Period.*

Duration of experiment, 74 days.

Nature of supplement.	Survival time.	Site and intensity of infection.	Growth response (in gm.).	Remarks.
0·04 mgm. carotene (*5 rats*)	To end of experiment	*Nil*	56–126 (average) (125% increase)	Perfectly healthy.
0·16 mgm. carotene (*9 rats*)	Ditto	„	59–139 (average) (136% increase)	„ „
0·5 gm. dried cabbage (*5 rats*)	„	„	57–133 (average) (133% increase)	„ „
10 mgm. cod-liver oil	„	Mastoids	54–89	General condition good.
Ditto	„	Mastoid	53–120	„ „
„	„	M.E.+	61–135	„ „
„ (*2 rats*)	„	*Nil*	58–128 (average for C.L.O. group 119% increase)	Perfectly healthy.
Nil	61	X. +; panophthalmitis (L.); T.A.+; bladder; kidney; pelvis	68–125–102	...
„	52	T.A.++	44–59–56	...
„	53	X.+; T.A.+; M.E. ++; bladder (with extreme hyperplasia)	56–77–56	...

were found, although loss of weight was evident. It is possible that a septicæmia may have been present in these cases without local foci, but on this point we have no evidence. If the generally accepted test of vitamin A be examined, namely, the property of causing growth resumption for a period of 4 weeks after A depletion, it will be seen that the statement of Euler *et al.* (1928) and Moore (1929), that amounts of the order of 0·005 mgm. of carotene are efficient, may be accepted. It is obvious, however, that the degree of protection against infection brought about by this quantity of carotene is only slight, and that ultimately both loss of weight and death with infective foci result. In other words, the protective dose of carotene is higher than that required to stimulate

TABLE V.—*Summary of Results showing Degree of Anti-Infective Action of Various Doses of Carotene.*

Experiments I and IV (No Depletion Period).

Protective agent.	Severe infection.	Moderate infection.	No infection.
None . . .	5	1	0
0·005 mgm. carotene	1	2	0
0·010 „ „	0	3	0
0·040 „ „	0	0	5
0·160 „ „	0	0	9
0·500 gm. dried cabbage	0	0	5

Experiments II and III (Preliminary Depletion Period).

Protective agent.	Severe infection.	Moderate infection.	No infection.
None . . .	4	0	0
0·005 mgm. carotene	5	1	0
0·010 „ „	0	3	2
0·020 „ „	1	0	5
0·040 „ „	0	1	3
0·080 „ „	0	0	4
0·500 gm. dried cabbage	0	0	7

growth for a short period, the minimal amounts for the two purposes being roughly of the order of 0·02 mgm. (for anti-infective action) and 0·005 mgm. (for growth resumption). Comparative effects of these two quantities of carotene on growth can be seen in the composite curves of Fig. 1. One point of interest in these growth observations, to which attention may be drawn, is that even when no vitamin D was added to the diet, good growth was maintained for a period of 78 days in animals receiving 0·01 mgm. carotene.

We are still not in a position to give any explanation of the mode of action of either vitamin A or carotene in raising the resistance of the body to infection. An earlier discussion, it may be remembered, centred round the question as to which was the first phenomenon produced by lack of vitamin A—the epithelial hyperplasia or infection of the epithelium. We were interested to note that in cases where 0·01 mgm. of carotene was given and the animals developed local septic foci, there was no epithelial hyperplasia obvious to the naked eye in organs other than those infected, and possibly not in these. This may mean either that the hyperplasia had been brought back to normal by the carotene, or that some hyperplasia might still be present, but could only be seen by microscopic examination. Investigations into this and other related problems should now be accelerated, for in carotene we have a substance functioning as vitamin A whose dosage can be accurately controlled and which can be given free from complicating factors.

Euler, Euler and Karrer (1929) have recorded the presence of carotene in the liver after the feeding of carotene to rats. Presumably when storage

occurs the animal is receiving a sufficient supply of carotene for its immediate needs. It is significant in this respect that no carotene was found in the liver by these investigators when the daily dose was below 0·029 mgm., whilst a dose of under 0·005 mgm. was sufficient to restore growth (for the experimental period of 35 days). Our observations on the livers of carotene-fed animals suggest that it is stored, not as carotene, but as vitamin A, as recently indicated by Moore (1929), but we find that the minimal dose required to bring about liver retention is considerably higher than that suggested by Moore—at any rate in rapidly growing animals.

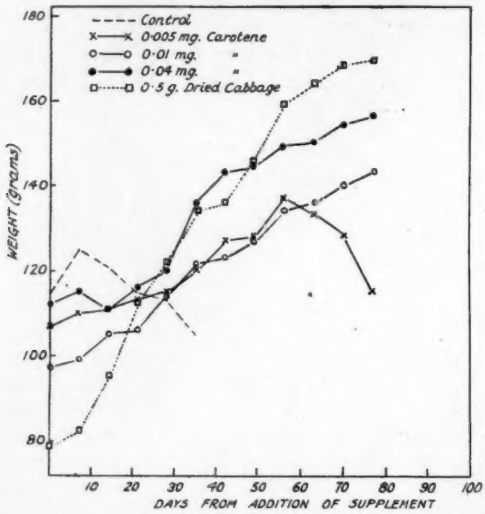

FIG. 1.—The effect of varying quantities of carotene on the growth of young rats after a depletion period (−A diet) of 32 days. Each curve is composite.

In our previous communications we assigned to vitamin A an anti-infective function, though we always realized that there might be other factors associated with it in the green part of the plant or in the liver oil which might play some subsidiary part in this protective function. The fact that carotene itself in sufficient dosage protects the animal completely against this spontaneous infection indicates that the effect is due in both cases to one chemical entity. Since, however, vitamin D, the only vitamin of which we have any quantitative knowledge, protects against rickets in doses as small as 0·0001 mgm.—a much smaller dose than is necessary in the case of carotene when used as an anti-infective agent—we hesitate to ascribe the specific effect definitely to carotene itself, and there is still a slight possibility that the active agent is an impurity—maybe a carotinoid derivative allied to the vitamin A compound as found in liver. The balance of evidence, however, favours the view that carotene is itself the specific substance, and that it is responsible for the vitamin A

properties of green vegetables, carrots and butter and probably egg-yolk. Euler *et al.* (1928) stated that the ether-soluble lipochrome extracted from human blood not only gives the characteristic blue colour with $SbCl_3$ but also the absorption bands of carotene. If this be so, there is a possibility that the liver stores its vitamin A as a highly active leuco form of carotinoid, which may be reconverted to carotene and liberated into the circulation as required.

If carotene has a similar function in man to that in the rat, as seems probable, it should prove valuable both as a prophylactic and therapeutic agent. As a therapeutic agent it should be of special importance for rapid and effective action, for it can be given without the large amount of lipoid material, often badly tolerated, which has to be given in the massive vitamin A therapy with liver-fat preparations.

Incidentally, these results suggest the desirability of a greater use of carrots where vitamin A is indicated because of their relatively large content of carotene as recently emphasized by Moore (1929).

SUMMARY.

(*a*) Tests on a specimen of carotene (melting-point 174°) showed that this substance had the property of conferring complete immunity in growing rats against the development of spontaneous infection.

(*b*) Animals on a diet free from vitamin A and carotene invariably developed septic foci and died. When carotene was given in the food the amount of protection conferred on the animals was generally proportional to the amount of carotene eaten.

(*c*) With the basal diet used in these experiments, carotene in amounts of 0·005 mgm. gave only slight immunity, 0·01 mgm. partial immunity, whereas 0·02 mgm. and greater amounts gave complete or practically complete immunity.

We are indebted to the Medical Research Council for the expenses of this investigation.

REFERENCES.

Carr, F. H., and Price, E. A.—(1925) *Biochem. J.*, **19**, 753.
Collison, D. L., Hume, E. M., Smedley-Maclean, I., and Smith, H. H.—(1929) *Ibid.*, **23**, 634.
Dulière, W. L., Morton, R. A., and Drummond, J. C.—(1929) *J. Soc. Chem. Ind.*, **48**, 518.
Euler, B. von, Euler, H. von, and Hellström, H.—(1928) *Biochem. Z.*, **203**, 370.
Euler, B. von, Euler, H. von, and Karrer, P. - (1929) *Ibid.*, **209**, 240.—(1929) *Helv. chim. Acta*, **12**, 278.
Green, H. N., and Mellanby, E.—(1928) *Brit. Med. J.*, **2**, 691.
Mellanby, E., and Green, H. N.—(1929) *Ibid.*, **1**, 984.
Moore, T.—(1929) *Biochem. J.*, **23**, 802.—(1929) *Lancet*, **2**, 380.

VARIATIONS IN THE PHOSPHATASE ACTIVITY OF THE BLOOD IN DISEASE.

W. MORRELL ROBERTS.

From the Department of Pharmacology, University of Sheffield.

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THE object of this paper is to describe a simple and comparatively rapid method for the approximate estimation of the phosphatase content of the blood, elaborated for an independent clinical investigation about which it is not proposed to speak here, and to report the results obtained on applying the method to the blood of patients suffering from a variety of conditions, particularly such types of case as might be expected to show some deviation from the normal value.

The principle employed was that used by Kay (1926) for the estimation of tissue phosphatase, who takes as a measure of the phosphatase activity the increase in organic phosphorus resulting from incubation of the tissue extract with an excess of glycerophosphate under suitable conditions of hydrogen ion concentration.

At the outset, it was thought that plasma or serum would be preferable to whole blood for this estimation, since the corpuscles contain not only phosphatase, but a hydrolysable phosphoric ester. In practice, however, it was found that satisfactory comparable results were obtained with whole blood, which was therefore used throughout the investigation, whereby less blood was required from the patient and the necessity for its centrifugalization avoided.

An important point was to determine the optimum pH for the hydrolysis. This was accomplished by incubation of a series of blood samples with glycerophosphate and progressively increasing amounts of decinormal caustic soda solution, the volumes being equalized by the addition of the requisite amounts of distilled water. This procedure was carried out on different specimens of blood, and the maximum hydrolysis was found to occur in each case when the proportion of alkali was 0·6–0·7 c.c. to every 2 c.c. blood, whilst the final pH of these mixtures in which the maximum hydrolysis occurred varied from 8·8 to 9·2. One of these tests is represented in Chart 1 (curve 1).

Two hours' incubation was found to be ample to establish the normality or otherwise of the blood samples examined. Chart 2 illustrates the effect of incubation time on the value obtained.

Method.

Blood is collected from a vein in the usual manner and clotting prevented by the addition of the *minimal quantity* of oxalate crystals necessary. Two c.c. is measured into 4 c.c. distilled water and the action of the enzyme stopped,

and the proteins precipitated by the addition of 4 c.c. of a 20% solution of trichloracetic acid. After it has stood for a few minutes the mixture is filtered through an ash-free filter-paper (Filtrate A). A second 2 c.c. blood is measured into a 10 c.c. stoppered cylinder, 2·3 c.c. distilled water added and the pH adjusted by the addition of 0·7 c.c. decinormal caustic soda solution. (These additions may be carried out at one operation by taking 3 c.c. of a mixture of decinormal soda and water in the above proportions.) One c.c. of a freshly prepared 1% solution of sodium beta-glycerophosphate is then added, and the

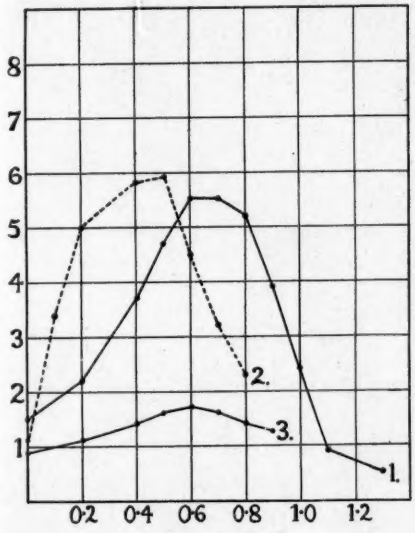

CHART 1.

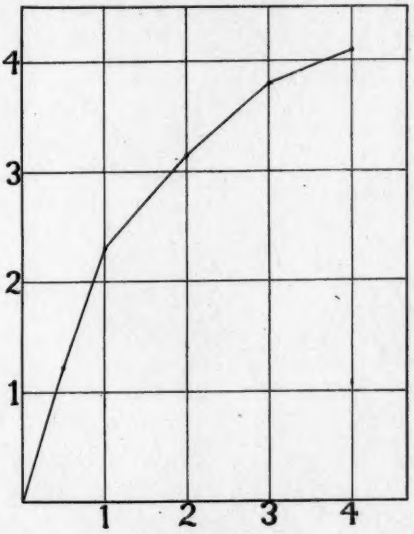

CHART 2.

CHART 1.—Showing the effect of increasing alkalinity on phosphatase activity of whole blood (curve 1); phosphatase activity of plasma (curve 2); hydrolysis of corpuscular hexose phosphate by phosphatase (curve 3). Ordinates = mg. phosphorus liberated per 100 c.c. blood or plasma. Abscissae = c.c. decinormal soda per 2 c.c. blood.

CHART 2.—Showing the effect of incubation time on the degree of hydrolysis. Ordinates = mg. phosphorus liberated per 100 c.c. blood. Abscissae = time of incubation in hours.

mixture incubated in a water bath at 38–40° C. for 2 hours. At the end of this time 4 c.c. of trichloracetic acid solution is added as before and the liquid filtered (Filtrate B). Alternatively, the two protein precipitations may be performed with 2 c.c. 40% trichloracetic acid, and after the addition of a trace of caprylic alcohol to disperse froth, the volumes made up to 10 c.c. with water. Identical results were obtained by the two procedures, and the former was adopted. The inorganic phosphorus is determined in aliquot parts of the filtrates by any suitable method. In this work Benedict's method, as described in Myer's 'Practical Chemical Analysis of Blood' was used, in which case a small correction is required for the hydrolysis of glycerophosphate by

TABLE.

Number of case.	Type of case.	Units of phosphatase.	Inorganic phosphorus.	Hexose phosphate.
1	Normal	4·5	8·2	1·1
2	„	3·8	7·9	1·5
3	„	3·0	7·8	0·9
4	„	4·1	7·5	0·7
	Malignant Disease.			
5	Carcinoma, stomach (secondaries in liver)	5·2	10·0	1·7
6	Secondary lung carcinoma	4·7	8·4	1·0
	Nitrogen Retention.			
7	Enlarged prostate (N.P.N. 50)	7·1	6·5	...
8	„ „ (N.P.N. 60·6)	6·0	8·1	2·4
9	„ „ (N.P.N. 55 9)	4·1	7·8	1·3
10	Chronic interstitial nephritis (N.P.N. 170·2)	6·8	11·8	0·2
	Anæmia.			
11	Pernicious anæmia	3·8	6·7	0·8
12	„ „	6·9	6·6	0·3
13	„ „	5·7	7·1	1·0
14	Secondary „	7·7	5·1	1·3
15	„ „	3·3	7·7	1·1
16	„ „	6·8	5·2	0·0
17	„ „ (Wassermann positive)	15·1	5·1	1·1
	„ „ (a month later)	19·0	4·4	1·0
	Osteitis Deformans.			
18	Osteitis deformans	17·2	6·2	2·0
19	„ „	28·2	8·0	4·9
20	„ „	19·2	8·0	4·4
21	„ „	8·2	7·2	0·9
	„ „ (a few weeks later)	12·3	7·6	0·9
	„ „ („ „)	12·4	7·5	1·9
22	„ „	15·8	8·1	1·1
	„ „ (2 weeks later)	18·9	7·7	3·5
	„ „ (1 week later)	18·5	8·1	...
23	„ „	24·0	8·3	2·9
24	„ „	32·1	9·0	3·1
25	„ „	21·8	7·4	3·5
26	„ „	6·5	8·5	...
27	„ „	31·4	7·1	...
28	„ „	21·2	6·0	...
29	„ „	10·1	7·4	...
30	„ „	18·0	...	...
	Bone Lesions other than Osteitis Deformans.			
31	Fracture, tibia and fibula (3 days)	6·5	6·6	0·7
32	„ „ (7 „)	7·8	7·4	1·2
33	„ „ (10 „)	8·4	8·4	1·2
34	„ „ (5 „)	7·4	8·0	1·0
35	Old fracture, radius; operated on 26 days previously, uniting	3·7	8·5	1·2
36	Fracture, spine (+ ? early osteitis deformans by X-ray)	4·2	8·4	1·8
	„ „ (a few days later)	3·3	8·2	1·5
	„ „ (2 months later, spinal fracture healed)	3·8	7·9	...
37	„ femur; 3 weeks (+ early osteitis deformans by X-ray)	6·2	8·6	0·7
38	„ „ 2 weeks (+ ? early osteitis deformans by X-ray)	3·3	7·6	...
39	Adolescent rickets	13·2	7·4	2·4
40	Healed rickets	4·5	7·2	1·2

Number of case.	Type of case.	Units of phosphatase.	Inorganic phosphorus.	Hexose phosphate.
	Diabetes.			
41	Diabetes (before insulin, blood-sugar 0·25)	6·0	6·6	2·7
	„ (a few hours later, after insulin, blood-sugar 0·08)	6·5	7·4	1·1
42	„ (on insulin)	4·8	...	...
43	„ (before insulin, blood-sugar 0·26)	6·9	6·2	0·3
	„ (after 2 days, insulin, blood-sugar 0·14)	11·4	6·7	1·2
	„ (after 8 days' insulin, blood-sugar 0·16)	6·5	6·8	1·1
44	„ (blood-sugar 0·34)	5·9	6·9	...
45	„ (on insulin, blood-sugar 0·15)	4·1	7·8	...
46	„ (before insulin, blood-sugar 0·20)	3·1	7·7	0·1
	„ (after 8 days' insulin, blood-sugar 0·10)	5·0	8·3	1·2
47	„ (before insulin, blood-sugar 0·26)	4·8	6·6	0·9
	„ (after few days' insulin, blood-sugar 0·15)	3·7	7·6	0·0
48	„ (coma; died few hours later)	12·3	8·7	...
	Jaundice.			
49	Obstructive jaundice (carcinoma pancreas; 12 units bilirubin)	18·5	6·4	1·1
50	„ „ (carcinoma stomach, secondaries liver; 15 units bilirubin)	19·6	6·5	...
51	Catarrhal jaundice (5 units bilirubin)	6·7	6·4	...
	Miscellaneous.			
52	Convalescent lobar pneumonia	3·5	7·9	0·8
53	Gastric ulcer	4·5	4·5	0·5
54	„ „	5·7	6·8	0·3
55	„ „	2·8	8·5	...
56	Duodenal ileus	2·9	8·4	1·0
57	Myxœdema	5·0	7·4	1·6
58	Exophthalmic goitre	5·7	7·1	0·7
59	Hæmoptysis (pulmonary tuberculosis)	4·2	7·4	0·8
60	Chronic bronchitis	3·0	8·1	0·8
61	Arterio-sclerosis	5·2	8·3	2·0
62	Myocarditis (Wassermann positive)	5·1	6·8	1·0
63	Aortic aneurysm (Wassermann positive)	7·0	6·8	1·1
64	Hodgkin's disease	5·4	7·9	0·4
65	Rheumatoid arthritis	6·9	8·1	1·4
66	„ „	6·7	8·1	1·7
67	Eczema (old-standing case)	5·4	6·8	0·6
68	Acne vulgaris	4·8	7·1	...

The inorganic phosphorus is expressed as milligrammes phosphorus per 100 c.c. blood. The mode of arrival at the hexose phosphate figures is described in the text.

the acid reagents during the heating in the boiling water-bath. This correction is determined by carrying out a blank estimation with glycerophosphate and reagents alone, and is about 0·6 units, as defined below, when pure glycerophosphate is used. The value so obtained is deducted from that found for filtrate B. The difference between B and A then represents the degree of phosphatase activity, when the latter is expressed as units of phosphatase per 100 c.c. blood, where one unit is the quantity of enzyme which liberates 1 mgm. phosphorus from excess of glycerophosphate in 2 hours under the above

conditions of hydrogen ion concentration, *i. e.* 8·8–9·2, as determined by the capillator.

Repeats agree well, but it is not claimed that this method gives more than an approximate measure of the phosphatase present. For accurate estimation a more elaborate method of fixing the pH is required, such as that described by Kay (1929), whose paper on the subject appeared whilst this work was in progress. The greater latitude allowed to the pH in the present method may lead to suboptimal conditions for hydrolysis in a few cases, when lower values will be found for the phosphatase than would have resulted from ideal conditions. On the other hand, this method is completed within 3 hours, whereas in Kay's method the incubation alone requires 48 hours.

When plasma was used for the estimation lower results were obtained than for whole blood, which suggested that corpuscles contain a larger proportion of enzyme than does plasma. But when plasma was incubated with different proportions of alkali in the manner described for whole blood, it became clear that the lower results were due to the fact that plasma is inferior to whole blood in buffering properties. The result is shown in Chart 1 (curve 2), where the optimum quantity of alkali is seen to be 0·4 c.c. In order to apply the method to plasma, it is necessary, therefore, to take 2·6 c.c. distilled water and 0·4 c.c. decinormal soda.

The results obtained on a specimen of blood were as follows :

	Decinormal soda.	Units phosphatase.
1. Whole blood	0·4 c.c.	5·3
2. „ „	0·7 „	9·9
3. Plasma	0·4 „	11·2
4. „	0·7 „	8·3

indicating that the greater proportion of phosphatase is present in plasma.

DISCUSSION OF RESULTS.

The whole blood phosphatase found in a variety of conditions is shown in the table (column 1), the inorganic phosphorus being recorded in column 2. The figures in column 3, referred to as "hexose phosphate," were obtained by incubation of the blood under identical conditions to those of the phosphatase estimation, except that the glycerophosphate was omitted, 1 c.c. of distilled water being substituted. The hexose phosphate figures depend essentially on two factors:

(1) The phosphatase activity of the blood,
(2) Its phosphoric ester content.

Hence, when the phosphatase activity is taken into account they may be regarded as giving some indication of the quantity of hydrolysable ester present. The effect of increasing alkalinity on this hydrolysis is shown in Chart 1 (curve 3).

As regards the phosphatase figures themselves, any value not exceeding 5·5 may be accepted as normal, the usual range being from 3 to 5 units.

The most striking feature of the series is the high values obtained in the thirteen proved cases of osteitis deformans (Paget's disease), the higher values occurring in the more advanced cases. This observation is in agreement with

that of Kay (1929), who found an increase of phosphatase in five cases of the disease.

High values were also obtained in the one case of adolescent rickets examined and in canine rickets as compared with controls, whereas healed human rickets gave a normal figure. The values in the cases of canine rickets are not included in the table. There was a tendency towards an increase in most of the cases of healing fractures, in two cases of rheumatoid arthritis and in conditions in which nitrogen retention was a feature.

The phosphatase was increased in most of the cases of anæmia. This is due to some extent to the higher phosphatase content of the plasma, the volume of which is increased relatively to that of the cells in anæmia, but hæmocrit measurements together with phosphatase estimations on whole blood and plasma showed that the increase could not be wholly accounted for on these grounds. The exceptionally high value in Case 17 also remains unexplained. This patient was examined clinically and was X-rayed for evidence of bone disease, but no abnormality of this nature was discovered.

There was a pronounced increase in phosphatase in obstructive and catarrhal jaundice. This is probably nothing more than an expression of the presence of bile constituents in the blood, since bile itself has a high phosphatase content.

An attempt was made to influence the phosphatase value by therapeutic means in two of the cases of osteitis deformans (Nos. 22 and 24), but no effect was observed to follow short courses of—

(1) Calcium-rich diet, with cod-liver oil and radiostol.

(2) Colloidal calcium and " Ostelin " vitamin D injections daily for 10 days, followed by injections at longer intervals.

(3) Calcium glycerophosphate gr. x *t.d.s.*

(4) Calcium glycerophosphate gr. x with sodium dihydrogen phosphate gr. xxx *t.d.s.*

(5) Calcium lactate gr. v *t.d.s.*

(6) Parathyroid and calcium tablets (Parke Davis); one tablet *t.d.s.*

SUMMARY.

1. A simple method for the estimation of blood phosphatase is described.

2. The enzyme is present in greater concentration in the plasma than in the corpuscles.

3. The blood phosphatase is markedly increased in osteitis deformans. It is raised also in active rickets and in obstructive jaundice, and to a more variable and slighter extent in certain other conditions.

4. Various therapeutic measures were without effect on the phosphatase level in osteitis deformans.

I would like to thank Prof. E. Mellanby for the interest he has taken in this work, and the members of the Honorary Staff of the Sheffield Royal Infirmary for bringing cases of Paget's disease to my notice. I have also to acknowledge the receipt of a grant from the Medical Research Council.

REFERENCES.

KAY, H. D.—(1926) *Biochem. J.*, **20**, 791.—(1929) *Brit. J. Exp. Path.*, **10**, 253.

A STUDY OF VACCINAL IMMUNITY IN RABBITS BY MEANS OF *IN VITRO* METHODS.*

S. R. DOUGLAS AND WILSON SMITH.

From the National Institute for Medical Research, Hampstead, London.

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IT has been shown by one of us (Smith, 1929) that vaccinia virus inoculated into a rabbit, either intravenously or intradermally, rapidly enters into a close association with some of the formed elements of the blood, most probably the white cells.

It was felt that this association between virus and blood-cells was unlikely to be a purely fortuitous one, that it might rather represent one factor in the first line of defence on the part of the host against parasitic invasion, analogous to the phagocytosis and subsequent destruction of bacteria by leucocytes. We were further encouraged in this assumption by the results obtained by Smith when he inoculated virus into immune rabbits: the virus was fixed by the blood-cells as in the case of the normal animal, but then rapidly disappeared from the circulation. It can scarcely be doubted that much, if not all, of such virus is actually destroyed somewhere within the immune organism, but little is known of the site and mechanism of such destruction. In the hope of gaining some information on these and allied questions the following research was undertaken. It was decided to use *in vitro* methods so as to reduce unknown factors to a minimum.

EXPERIMENTAL METHODS.

Many of the methods employed were identical with those used by Smith (1929) in earlier work; thus the reader is referred to his paper for details regarding the strain of virus, the withdrawal of blood samples, technique of blood fractionation and test inoculations.

In the following experiments the test materials were put into test-tubes, which were hermetically sealed with rounded ends in the blow-pipe flame, and then rotated on a revolving drum in a hot room at 37° C. for varying periods of time. The number of revolutions was adjusted to about 16 per minute, the test-tubes being inverted twice during each revolution, thus ensuring a continuous and thorough mixing of the contents.

I. EXPERIMENTS WITH NORMAL BLOOD.

A. *Can Virus Fixation be Demonstrated* in vitro?

A few experiments were carried out in order to ascertain whether the white blood-cells retain their power of virus fixation in blood withdrawn from the

* Part of this work was included in a thesis submitted by one of us (W.S.) for the M.D. degree of the University of Manchester.

circulation. Heparin (2 gm. per litre) was used as an anticoagulant. In each experiment 1 c.c. of a certain dilution of vaccinal testis diffusate or vaccinal testis emulsion was added to 9 c.c. of heparinized normal rabbit blood. After rotation of the mixtures for 3 hours at a temperature of 37° C. the blood was fractionated into plasma and washed cells, and each fraction was tested for the presence of virus by the intradermal inoculation of 0·2 c.c. into the epilated skin of the back of a normal rabbit. In some experiments the whole blood and a suspension of washed red blood-corpuscles freed from white cells by cotton-wool filtration (Fleming, 1926) were also tested.

The results shown in Table I indicate that a fixation of virus by the cellular elements of normal blood occurs *in vitro* similar to that which occurs *in vivo*. The red blood-corpuscles take no part in this process. In two experiments the whole of the virus present was apparently removed from the plasma and fixed by the cells. In the other two cases in which a larger quantity of virus was used, some of the virus remained in the plasma, but judging from the sizes of the resultant lesions most was taken up by the cells. It is obvious, of course, that a given number of cells cannot possess an unlimited power of virus fixation.

TABLE I.—In vitro *Fixation of Virus by Normal Blood-Cells.*

Virus material and final dilution.	Test rabbit No.	Results of test inoculations of blood fractions.			
		Whole blood.	Plasma.	Washed cells.	Washed and filtered red blood-corpuscles.
Testis diffusate (1 in 10)	881	+	−	+	−
„ „ (1 in 100)	881	±	−	+	−
„ emulsion (1 in 500)	888	...	+	+++	...
„ diffusate (1 in 10)	888	...	+	+++	...

N.B.—In all the tables the number of + signs indicates the comparative size of a reaction.

The next experiment was devised to find the time required for virus fixation. 18 c.c. of blood were withdrawn into the requisite amount of heparin solution; 3 c.c. of vaccinal testis diffusate were added, and mixed thoroughly at once by shaking. The mixture of virus and blood was then divided into four equal portions which were put into test-tubes, and the four tubes put on the rotating drum at 37° C. After ½ hour the first tube was taken and the contents fractionated and inoculated into the test rabbit. The other tubes were similarly treated at the end of 1 hour, 2 hours and 4 hours. All inoculations were made into the same test rabbit.

Each of the four mixtures gave precisely the same result:

Whole blood	++ reaction
Plasma	+ „
Washed cells	++ „

As the reactions given by the ½-hour sample appeared at the same time, and were the same size as those given by any of the later samples, it would appear that ½ hour is quite sufficient time for complete fixation to occur. In this experiment, however, an excess of virus was present, as shown by the positive reactions produced by all the plasma fractions. A second experiment

was therefore carried out using a much smaller dose of virus. In this experiment only the ½-hour and 4-hour samples were tested, with the following result:

	½-hour sample.	4-hour sample.
Whole blood	+	—
Plasma	±	—
Washed cells	—	—

This shows that with only a small amount of virus present fixation is very incomplete in ½ hour, so much so that the washed cells alone failed to give rise to any reaction. The absence of all virus in the 4-hour sample suggests either that the blood had exerted a viricidal action, or that the virus had died off by that time.

B. *Does the Plasma Play Any Part?*

It is well known that in the phagocytosis and digestion of bacteria by the leucocytes of both normal and immune blood the fluid constituents of the blood play a very important part. If the virus-fixation by leucocytes is a preliminary to ingestion and destruction, one would expect the serum constituents to play a part in the process parallel to the part they play in the destruction of bacteria.

The first step taken was to ascertain if the blood-cells could fix virus in the entire absence of plasma or serum. 4·5 c.c. of blood from a normal rabbit was withdrawn into 0·5 c.c. of heparin solution and then centrifuged until the cells were tightly packed down. The plasma was pipetted off and discarded. The cells were washed six times in physiological saline and were finally resuspended in saline to the original volume. 0·5 c.c. of vaccinal testis diffusate was added to 4·5 c.c. of the saline suspension of blood-cells and the mixture rotated at 37° C. for 3 hours. After rotation a sample was removed and the remainder fractionated into supernatant fluid and cells, the latter being washed 4 times before resuspension to the original volume. Each of the fractions and also the sample of the non-fractionated mixture were tested for virus, both undiluted and diluted 1 in 10.

This experiment was repeated using a smaller quantity of virus (0·25 c.c. of vaccinal testis diffusate to 4·75 c.c. of cell suspension). The details and results are shown in Table II. The only conclusion which can be drawn from these experiments is that some virus fixation can occur in the absence of plasma or serum.

TABLE II.—*Fixation of Virus by Blood-Cells in Saline Suspension.*

Experiment No.	Virus added to blood-cells.	Time of rotation.	Test rabbit No.	Result of test inoculations.					
				Whole suspension.		Supernatant fluid.		Washed cells.	
				1/1.	1/10.	1/1.	1/10.	1/1.	1/10.
1	0·5 c.c. testis diffusate	3 hrs.	1000	+	++	+	+	++	+
2	0·25 c.c. testis diffusate	2¾ „	1002	+	+	+	+	+	+

An experiment was now carried out to compare directly the action upon virus of—

(*a*) Blood-cells without plasma: The saline suspension of cells was obtained in exactly the same manner as described above.

(*b*) Blood-cells with plasma: In order that the blood-cells in this mixture should be strictly comparable to those acting without plasma they were first separated by centrifugation, washed six times in saline and then resuspended in the plasma to form "reconstituted blood."

0·25 c.c. of a virus diffusate was added to 4·5 c.c. of both (*a*) and (*b*). Each mixture was rotated for 4 hours at 37° C., then fractionated and tested for virus in the usual manner, the fractions from (*a*) being inoculated into the skin of one side of the test rabbits, those from (*b*) into corresponding sites of the skin of the other side. The test inoculations were made in duplicate on two test rabbits; the resultant lesions were exactly the same in each case, and were as follows:

	Saline susp.	Reconstituted blood.
Whole suspension or whole blood . .	++	±
Supernatant fluid or plasma . .	+	−
Washed cells (washed 3 times) . .	++	±

From this experiment it would appear that plasma plays a very important *rôle* in the action of blood on virus. Virus fixation was possibly complete in its presence, but certainly incomplete in its absence. Furthermore, there is definite evidence of virus destruction by the reconstituted normal blood, for degradation of the virus by keeping for some hours at 37° C. would occur just as readily in a saline suspension of cells as in reconstituted blood. It should be mentioned that many experiments have shown that normal blood-plasma or serum, in the absence of blood-cells, exerts no destructive action on virus.

c. *Importance of a Thermolabile Constituent of Plasma or Serum.*

In order to test whether the action of the plasma depends upon a thermolabile constituent, normal blood-cells were obtained from heparinized blood, washed six times with physiological saline, and then resuspended to the original volume in—

(*a*) Untreated heparinized plasma.

(*b*) Plasma heated in a water-bath to 56° C. for ½ hour.

Equal quantities of virus were added to each of these cellular suspensions and the mixtures rotated for 3¼ hours, after which they were fractionated. The test inoculations of the blood fractions were again duplicated, and as the results in the two test rabbits differed slightly, both series are given:

Test rabbit No.		Whole blood.	Plasma.	Washed cells.
1043	Mixture with untreated plasma	−	−	+
	„ heated „	+++	+++	+
1044	„ untreated „	−	−	−
	„ heated „	+++	+++	+

It is quite clear that heating the plasma to 56° C. for ½ hour considerably reduced, if it did not destroy entirely, the viricidal power of the blood.

A similar experiment was now performed, using serum instead of plasma. In this experiment the blood-virus mixtures were not fractionated after rotation, but ten-fold dilutions of the whole mixtures were made in saline and 0·2 c.c. of each dilution inoculated into the test rabbit:

	Dilutions tested for virus.			
	1/1.	1/10.	1/100.	1/1000.
Mixture with untreated serum	++	+	−	−
„ heated „	++	+	−	−

This result suggested that the serum did not contain the important thermolabile constituent found in plasma. The serum used in the above experiment, however, was old stock serum which had been originally filtered through a Seitz filter and then kept for several months at a temperature ranging from 0° C. to 5° C. It was thought probable that prolonged storage might alter or destroy a thermolabile constituent, in which case its demonstration could not be expected in such old serum. The experiment, therefore, was repeated using freshly obtained and unfiltered rabbit serum. The portion heated was subjected to 58° C. for ½ hour, also the cells were washed only three times before re-suspension, as it was thought that the long treatment at room temperature and the severe mechanical strain consequent upon six washings might do damage to the viricidal blood-cells. The results, which need no further comment, are given below:

	Results of test inoculations of dilutions.					
	1/1.	1/10.	1/100.	1/500.	1/1000.	1/2000.
Mixture with untreated serum	++	+	−	−	−	−
„ heated „	+++	++	+	+	+	−

The experiment was again repeated. On this occasion not only were dilutions of the mixtures tested, but also part of each mixture was fractionated and the separate fractions tested. We reverted to six washings of the cells before resuspension and rotation and three washings of the cellular fraction after rotation. The heated serum was subjected to 57° C. for ½ hour, and the time of rotation was 2 hours. Duplicate test rabbits were used; the results from both are given:

Test rabbit No.		Washed cells.	Serum.	Whole blood dilutions.			
				1/1.	1/10.	1/100.	1/1000.
1109	Mixture with untreated serum	+	±	+	−	−	−
	„ heated „	++	++	+++	+	±	−
1110	„ untreated „	±	±	+	−	−	−
	„ heated „	++	++	+++	++	−	−

It was now decided to test the effect of a diluted serum, both heated and unheated, in order to ascertain if a very small quantity of the thermolabile constituent might suffice to enable the cells of normal blood to exert to the full their viricidal power. A fresh serum was therefore diluted 1 in 100 with saline and half of it heated to 57° C. for ½ hour. Washed cells were then

re-suspended in both the heated and unheated dilute serum, virus added in equal amounts to each tube, and the mixtures rotated for 1½ hours at 37°C. The results showed that more virus survived in the mixture with heated serum, but this was evidenced more by the sizes of the resultant skin lesions than by positive takes in higher dilution. It would appear that the actual quantity of thermolabile substance present is of importance in the viricidal action of the blood.

Results:

Test rabbit No.		Washed cells.	Serum.	Whole blood dilutions. 1/1.	1/10.	1/100.
1117	Mixture with untreated serum (1 in 100 dilution)	+	+	+	±	±
	„ heated „ ditto	+	++	++	+	±
1118	„ untreated „ „	+	++	++	+	−
	„ heated „ „	+	+++	+++	+	+

D. *The Effect of Different Anti-coagulants on Viricidal Power.*

It is well known that the addition of sodium citrate solution to blood, in amounts sufficient to prevent clotting for some hours, inhibits the phagocytic activity of the leucocytes against visible bacteria. Walsh and Harmsworth (1927) showed that hirudinized blood possessed much greater bactericidal power than citrated blood. Owing to a new batch of heparin proving unsatisfactory in its anticoagulant properties it was decided to compare the viricidal powers of blood mixed with heparin, sodium citrate and potassium oxalate, in the hope that one of the latter anticoagulants might be successfully employed in subsequent work. Four experiments were carried out, the details and results of which are given in Table III. In Experiments 1 and 2 the effects of heparin and sodium citrate were compared, in Experiment 3 heparin and potassium oxalate, and in Experiment 4 each of the anticoagulants was tested against both the others by rotating three mixtures side by side, and then using three test rabbits, thus eliminating fallacies arising from individual variations in susceptibility of rabbits to vaccinia virus.

The results show that sodium citrate exerts a considerable inhibiting effect upon the viricidal action of blood. Potassium oxalate would appear to be a suitable anticoagulant for experiments of this nature, but heparinized blood showed throughout the greatest viricidal power, and it was decided to retain its use in subsequent work.

In order to eliminate the possibility that heparin *per se* might exert a viricidal action, virus was rotated, both with and without heparin, in saline emulsion, and then tested by inoculating serial dilutions of the mixtures. In one experiment the heparinized virus "took" to exactly the same titre as non-heparinized virus; in another experiment the heparinized virus took actually in higher dilution.

II. EXPERIMENTS WITH BLOOD AND OTHER TISSUES OF IMMUNE ANIMALS.

A. *Is Immune Blood more Viricidal than Normal Blood?*

It has been mentioned above that virus inoculated into the blood-stream of an immune rabbit is taken up, at least in part, by the cells, but can only be

TABLE III.—*Effects of Various Anti-Coagulants on Viricidal Power.*

Exp. No.	Comparison of—	Virus added to blood.	Time of rotation in hours.	Test rabbit No.	Results of inoculations of dilutions.					
							1/1.	1/5.	1/25.	1/125.	1/625.
1	Heparin *v.* citrate 1·5%	Testis emulsion	2½	1144	Heparin mixture		+++	++	+	+	−
					Citrated	,,	+++	+++	++	++	+
2	Ditto	Ditto	3½	1150	Heparin	,,	+	+	−	−	−
					Citrated	,,	+++	+++	++	++	+
2	,,	,,	3½	1151	Heparin	,,	+	±	−	−	−
					Citrated	,,	+++	+++	++	+	+
3	Heparin *v.* oxalate 0·125%	,,	4½	1167	Heparin	,,	+++	+++	++	+	+
					Oxalated	,,	+++	+++	++	+	+
3	Ditto	,,	4½	1168	Heparin	,,	++++	+++	++	+	?
					Oxalated	,,	++++	+++	++	+	?
4	Heparin *v.* citrate 1·5%	,,	4	1182	Heparin	,,	++	++	+	+	−
					Citrated	,,	++++	+++	++	++	+
4	Heparin *v.* oxalate 0·125%	,,	4	1183	Heparin	,,	+++	++	+	±	±
					Oxalated	,,	+++	++	++	+	+
4	Citrate *v.* oxalate	,,	4	1184	Citrated	,,	+++	++	++	+	+
					Oxalated	,,	++	+	+	±	±

recovered by the fractionation technique for a few hours (Smith, 1929). It is not known what happens finally to such virus, whether the blood-cells destroy the virus *in situ*, or whether the cells containing virus migrate out from the blood-stream into internal organs, such as the liver and spleen, as a preliminary to virus destruction in those situations.

An experiment was planned to compare the effects of normal and immune blood on the virus *in vitro*. Two tubes were rotated together, one containing a mixture of virus and heparinized normal blood, the other containing a strictly comparable mixture of virus and heparinized immune blood. The immune blood was withdrawn from a rabbit which had recovered from generalized vaccinia following the intravenous inoculation of neurovaccine (Douglas, Smith and Price, 1929). A preliminary titration of the antibody content of plasma obtained from this animal showed that 1 c.c. was capable of neutralizing at least 10,000 minimal infective skin doses of virus. After 3 hours' rotation each mixture was fractionated, and the fractions tested by intradermal inoculation. The fractions from the normal blood-virus mixture were inoculated into one side of the back of the test rabbit, those from the immune blood-virus mixture into corresponding skin sites of the opposite side.

The results were as follows:

a. Fractions from normal blood-virus mixture:

Whole blood .	++
Plasma . .	+
Washed cells .	+++
Red blood-cells	−

b. Fractions from immune blood-virus mixture:

Whole blood .	−
Plasma . .	−
Washed cells .	−
Red blood-cells	−

As regards normal blood, the results confirm those of the previous experiments. The skin reaction resultant from the plasma inoculation was very slight and did not appear at all until the fourth day, at a time when the other positive reactions were well advanced, so that obviously the blood-cells had fixed by far the greater part of the virus present in the mixture.

It is more difficult to interpret the results obtained with the immune blood fractions. There are at least three possible explanations:

(*a*) Immune cells may have fixed some or all of the virus and destroyed it—that is, immune blood-cells may be much more viricidal than normal cells.

(*b*) Antibodies in the immune plasma may have prevented the immune cells from taking up virus, or, conversely, may have prevented the virus from attacking the cells. In this case the virus, although present in the plasma at the end of rotation, would be "masked" by neutralizing antibodies in the usual manner.

(*c*) In spite of thorough washing, enough antibody may have remained attached to the immune cells to neutralize any virus taken

up by such cells. In other words, the virus may have been fixed by the cells, but not killed by them.

Of these possibilities the first is the most reasonable. Both the others are unlikely in view of Smith's *in vivo* experiments, in which by the use of the same fractionation technique, immune cells were shown to take up virus which had been inoculated intravenously.

Furthermore, an attempt was made to recover virus from the plasma by using the technique described by Andrewes (1928), by which he was able to recover virus constantly from inactive mixtures of virus and immune serum.

For this purpose 1 c.c. of the plasma was diluted 1 in 10 with distilled water, and the euglobulins precipitated by passing CO_2 through the solution. The precipitate was thrown down by centrifuging, and washed three times with distilled water saturated with CO_2. It was then re-dissolved in 1 c.c. of Ringer's solution, and tested for virus by the intradermal inoculation of 0·2 c.c. of the solution into the same test animal as was used for the testing of the various blood fractions. There was no sign of reaction at the site of this inoculation. The negative result of this experiment is strong evidence against the hypothesis of mere masking of virus in the plasma.

B. *Interchange of Cells and Plasma of Normal and Immune Blood.*

In Section I it was shown that, as regards fixation of virus by the white cells of normal blood, both cells and plasma play a part in the mechanism. Possibly both constituents are altered in the process of immunization, which apparently confers increased viricidal power. It was thought that information on this point might be obtained from experiments in which the cells and plasma of normal and immune blood were interchanged.

Experiment.

18 c.c. of blood from immune rabbit 875 were withdrawn into 2 c.c. heparin solution, and the mixture separated into plasma and cells, the latter being washed three times in saline. 18 c.c. of blood from a normal rabbit were treated in exactly the same way. The following blood mixtures were then prepared:

(A) 1 vol. washed immune cells + 1 vol. immune plasma (*i.e.* reconstituted immune blood).
(B) 1 „ normal „ + 1 „ normal „ („ normal „).
(C) 1 „ immune „ + 1 „ „ „
(D) 1 „ normal „ + 1 „ immune „

To each of the four blood mixtures 1 c.c. vaccinal testis diffusate was added. The tubes were sealed and rotated for 4½ hours at 37° C. After rotation the mixtures were fractionated and the fractions tested for virus. Two test animals were used, one receiving inocula derived from A and B, the other inocula derived from C and D.

The results shown in Table IV confirm those of preceding experiments with regard to the viricidal action of the reconstituted normal and immune blood. They also apparently indicate that the immune bodies in plasma play a very important part in the destruction of virus by the cells. Normal cells in

the presence of immune plasma were more destructive for virus than immune cells in the presence of normal plasma. A possible explanation of this would be an opsonic or protryptic power of the immune plasma antibodies, similar to the protryptic power of serum for bacteria—Douglas (1916)—rendering the virus more susceptible to ingestion and destruction by the cells.

TABLE IV.—*Effect of Interchange of Cells and Plasma of Normal and Immune Blood.*

Test rabbit No.	Nature of blood mixture.	Results of inoculations of blood fractions.		
		Whole blood.	Plasma.	Washed cells.
912	Reconstituted normal blood	+++	+	+++
	„ immune „	−	−	+
911	Immune cells + normal plasma	++	+	++
	Normal „ + immune „	−	−	+

The employment of the two test animals in such an experiment is open to the objection that any comparison of reactions on different rabbits is invalid. The experiment was therefore repeated, and all the test inoculations made on the same rabbit. The results are presented in Table V; they show that, compared with reconstituted normal blood, each of the other blood mixtures showed greater viricidal activity. The washed cells from the immune cell-normal plasma mixture produced an earlier and slightly larger reaction than those from the normal cell-immune plasma mixture, thus again suggesting the possibility of an opsonic or protryptic factor of immune plasma playing some part in the viricidal mechanism.

TABLE V.—*Effect of Interchange of Cells and Plasma of Normal and Immune Blood.*

Nature of blood mixture.	Results of inoculations of blood fractions.		
	Whole blood.	Plasma.	Washed cells.
Reconstituted normal blood	+++	±	+++
„ immune „	±	−	±
Immune cells + normal plasma	+	−	+
Normal „ + immune „	−	−	+

N.B.—All the inoculations were made on test rabbit No. 925.

c. *Investigation of the Viricidal Power of the Immune Cell* per se.

The investigations of Andrewes (1928) have shown that serum antibodies against vaccinia virus, although possessed of neutralizing power *in vivo*, do not actually kill the virus *in vitro* within 24 hours. It seemed to us improbable that the greatly increased viricidal power of the immunized animal could depend entirely upon such antibodies, and many experiments were carried out in

order to determine whether the immune cell itself has acquired new properties conferring an increased viricidal power. In view of the importance of the reticulo-endothelial system in the mechanism of protection against bacterial invasion, tissue suspensions were tested in some of the experiments instead of blood-cell suspensions. Both spleen and testis were used because it was felt that if cellular immunity apart from humoral immunity is a reality it might be shown more clearly in one tissue-cell than in another.

The first experiment was a direct comparison of the viricidal activities of normal and immune blood-cells, washed three times to remove serum antibodies and resuspended in saline to the original volume.

Two tubes, A and B, were rotated for 2½ hours at 37° C.

A = Washed cells of normal blood suspended in saline + virus.
B = „ „ immune „ „ „ + „

After rotation five-fold dilutions of each mixture were made and tested for virus by intradermal inoculation, with results as follows:

	Dilutions of mixtures tested.			
	1/1.	1/5.	1/25.	1/125.
Normal cells + virus	+++	++	+	+
Immune cells + virus	+	−	−	−

In order to ascertain whether the virus remaining in the immune cell mixture was fixed to the cells or left free in the fluid menstruum, a similar experiment was done in which fractionation after rotation was practised.

Two tubes were rotated for 2 hours at 37° C.:

A = Washed normal cells (4 washings) suspended in saline + virus.
B = „ immune „ „ „ „ + „

After rotation the mixtures were centrifuged, the supernatant fluids removed, the cells again washed three times and resuspended in saline. Ten-fold dilutions of both cell suspensions and supernatant fluids were then tested for virus.

Result:

Fractions tested.	Fraction obtained from—	Dilutions tested. 1/1.	1/10.	1/100.	1/1000.
Cells	Normal cell suspension mixture	++	+	+	−
	Immune „ „	−	−	−	−
Supernatant fluids	Normal „ „	+++	++	+	+
	Immune „ „	+	+	+	+

Suspensions of washed spleen cells were tested by an experiment similar to those just described.

Rabbit 863, which had received several intradermal injections of virus on 15 . 10 . 28, was killed on 3 . 12 . 28. 1 gm. of the spleen tissue was minced with scissors and emulsified by grinding in a sterile mortar with 10 c.c. of physiological saline. 8 c.c. of the emulsion were centrifuged, the supernatant

fluid was discarded, and the cellular deposit washed twice prior to resuspension in saline to the original volume of 8 c.c.

1 gm. of spleen from a normal rabbit was treated in exactly the same way.

To each of the spleen cell suspensions 2 c.c. of vaccinal testis diffusate were added. The mixtures were rotated side by side for 3 hours at 37° C. After rotation both mixtures showed some coarse particles, which were allowed to sediment. Dilutions of the suspensions were then tested for virus by intradermal inoculation:

Dilution tested.	Normal spleen.	Immune spleen.
Undiluted	++	+
1/10 dilution	+	+
1/100	+	—
1/1000	+	—

The experiments so far recorded suggest that there is an inherent difference between the normal and immune cells in the case of both blood and spleen. There remains, however, the possibility that the apparently greater viricidal power of immune cell suspensions may depend upon the neutralizing activity of antibody, which remains attached to the cell surfaces in spite of repeated washing with saline. In order to remove this possible source of error experiments were devised in which the final cell-virus mixtures to be compared should be bound to contain exactly equal quantities of antibody, either sessile or free. These experiments differed only in minor details, which are shown in the respective tables; the scheme shown in the figure applies to all of them.

It will be seen that the final mixtures to be compared by inoculation contained exactly the same ingredients, the only difference being that in the mixture A + D the virus had been acted upon by *normal* tissue cells during the period of rotation, whilst in the mixture B + C the virus had been acted upon by *immune* cells.

The first experiment based upon this scheme was a comparison of normal and immune blood-cells. The four mixtures were:

A = 4 c.c. normal-blood cells suspended in 1 in 5 dilution of normal plasma + 0·5 c.c. vaccinial testis emulsion (1 in 100 dilution centrifuged) + 0·5 c.c. saline.

B = 4 c.c. normal blood-cells suspended in 1 in 5 dilution normal plasma + 0·5 c.c. saline.

C = 4 c.c. immune blood-cells suspended in 1 in 5 dilution immune plasma + 0·5 c.c. vaccinial testis emulsion + 0·5 c.c. saline.

D = 4 c.c. immune blood-cells suspended in 1 in 5 dilution immune plasma + 0·5 c.c. saline.

The rotation time was 2½ hours.

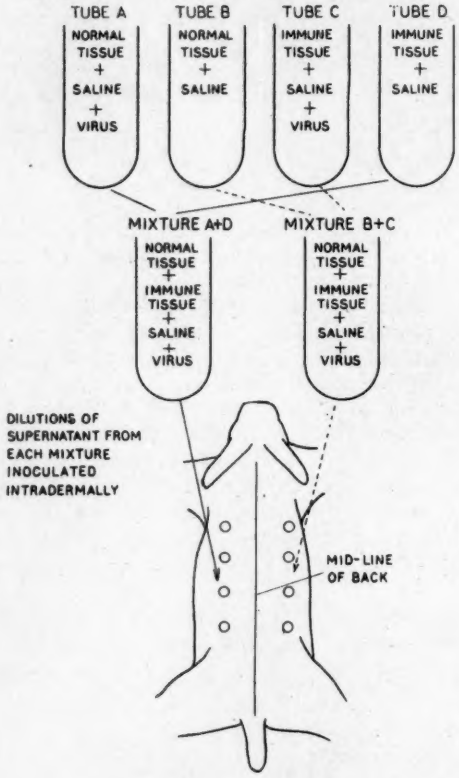
Result:

	Dilutions of mixtures tested.				
	1/1.	1/5.	1/25.	1/125.	1/625.
Mixture A + D	+	++	++	?	±
Mixture B + C	+	—	—	—	—

The larger reactions given by the 1 in 5 and 1 in 25 dilutions of mixture A + D are interesting as they probably represent the dilution phenomenon of Todd (1928).

Four experiments were now carried out in which the spleens and testes from normal and immune rabbits were used in place of blood-cells.

In every case duplicate test rabbits were used, but as these never gave


TUBE A
NORMAL TISSUE + SALINE + VIRUS
TUBE B
NORMAL TISSUE + SALINE
TUBE C
IMMUNE TISSUE + SALINE + VIRUS
TUBE D
IMMUNE TISSUE + SALINE
MIXTURE A+D
NORMAL TISSUE + IMMUNE TISSUE + SALINE + VIRUS
MIXTURE B+C
NORMAL TISSUE + IMMUNE TISSUE + SALINE + VIRUS
DILUTIONS OF SUPERNATANT FROM EACH MIXTURE INOCULATED INTRADERMALLY
MID-LINE OF BACK

contradictory results duplication is omitted from Table VI, which shows the details and results of all four experiments.

These results suggest very strongly that the immune animal possesses tissue-cells modified in some way which enables them to deal more effectively with the invading virus. It must be freely admitted, however, that there is no absolute proof of this; we have so far been unable to devise an experiment with conditions beyond criticism. The most striking results are shown in the experiments where the blood or tissue-cells were not washed before resuspension and rotation. This may be due to mechanical injury of the cells by the washing,

TABLE VI.—*Increased Viricidal Power of Tissue-Cells Derived from Immunized Animals.*

Dilutions tested.

Time of Mixtures

TABLE VI.—*Increased Viricidal Power of Tissue-Cells Derived from Immunized Animals.*

Exp. No.	Tissue used.	Treatment of tissue before suspension in saline.	Virus added to tubes A and C.	Suspension fluid.	Time of rotation in hours.	Mixtures tested.	Dilutions tested. 1/1.	1/5.	1/25.	1/125.	1/625.
1	Spleen	Ground up with saline, centrifuged cells washed 3 times	0·5 c.c. R. 1135 testis emulsion 1/250 dilution; centrifuged	Saline	2	A + D	+ + + +	+ + +	+ +	+	±
						B + C	+ + +	+ +	+ +	+	±
2	„	Spleen minced fine, with scissors; no preliminary washing	0·5 c.c. R. 1135 testis emulsion 1/200 dilution; centrifuged	„	2	A + D	+ + + +	+ + +	+ +	+	+
						B + C	−	−	−	−	−
3	Testis	Testis minced; no preliminary washing	Ditto	„	3½	A + D	+ + + +	+ + +	+ +	+	±
						B + C	+ +	+	−	−	−
4	„	Testis minced; no washing	0·5 c.c. R. 1135 testis emulsion 1/100 dilution; not centrifuged	„	3	A + D	+ + +	+	−	−	−
						B + C	−	−	−	−	−

TABLE VII.—*Action of Immune Serum on Viricidal Power of Normal Tissue-Cells.*

Exp. No.	Tissue employed.	Virus added to each system.	Amount of immune serum added to each system.	Time of rotation in hours.	Test rabbit No.	Mixtures tested.	Dilutions. 1/1.	1/5.	1/25.	1/125.	1/625.	1/3125.
1	Normal testis	0·5 c.c. R. 1135 testis emulsion 1/100 dilution; centrifuged	1 c.c.	4	1169	A	−	−	−	−	−	−
						B + C	−	+	−	−	−	−
					1170	A	+	+	−	−	−	...
						B + C	+	+ +	−	−	−	...
2	„ „	0·5 c.c. R. 1135 testis emulsion 1/100 dilution; not centrifuged	1 „	3	1230	A	+ +	+	+	−	−	...
						B + C	+ + +	+ +	+	−	−	...
					1231	A	+	−	−	−	−	...
						B + C	+ +	+ +	−	−	−	...
3	„ spleen	Ditto	1 „	4	1173	A	+ +	+ +	+ +	+	−	...
						B + C	+ +	+ +	+ +	+ +	+	...
					1174	A	+ +	+ +	+	+	−	...
						B + C	+ +	+ +	+	+	−	...
4	„ blood	„	1 „	4½	1180	A	−	−	−	−	−	...
						B + C	+	−	−	−	−	...
					1181	A	+	−	−	−	−	...
						B + C	+	−	−	+	−	...
5	„ „	0·5 c.c. R. 1199 testis emulsion 1/50	1 „	3	1213	A	+	+ +	+	−	−	...
						B + C	+ +	+ +	+	±	−	...
					1214	A	+	+	−	−	−	...
						B + C	+	+ +	+	−	−	...

A = tissue + virus + immune serum. B = tissue + virus. C = immune serum.

but it may equally well be due to an unknown function of immune bodies of the serum, quite other than the well-known power of neutralization of virus. The conditions of the experiments certainly equalize the antibody content of the two final mixtures compared, but they do not equalize other possible actions of immune serum. Thus it will be seen by a reference to the general scheme of the experiments that whereas the immune cells are acting upon virus *in the presence of* any neutralizing antibodies which may arise from the minced tissue, the normal cells are acting upon virus *in the absence of* such antibodies, which are only added in the final mixing shortly before inoculation. It was in the hope of finding out whether such an interpretation could be valid that the following investigations were made:

D. *Investigation of the* Rôle *of the Serum Antibodies.*

Five experiments were carried out, all on the following general plan. Three tubes were rotated together at 37° C. for varying lengths of time.

Tube A = Normal tissue + saline + virus + immune serum.
Tube B = Normal tissue + saline + virus.
Tube C = Immune serum.

After rotation B and C were mixed together. In experiments with spleen or testis the mixtures A and B + C were centrifuged and dilutions of the supernatant fluids tested, in the case of blood the whole mixtures were tested in five-fold dilutions without centrifugation. The details and results are shown in Table VII. In all experiments duplicate test animals were used; as these did not always agree the reactions of both are included.

The results are irregular; we think the chief reason for this is to be found in the dilution phenomenon of Todd (1928), for in all these experiments we were dealing with immune serum-virus mixtures. It is worthy of note, however, that in many cases greater viricidal action was obtained where the cells exerted their action in the presence of immune bodies, but never was an opposite result obtained. Whether this was due to an opsonic action of the antibodies or to a stimulation of the phagocytic cells, or to some other, as yet quite unknown, factor we are unable to say.

DISCUSSION.

With regard to the mechanism of the increased protection which immunized animals acquire against certain diseases due to filterable viruses, there has been a tendency of late years for investigators to range themselves in two opposing camps. There are some who hold that immunity reactions in virus diseases differ fundamentally from those which obtain in diseases produced by the visible bacteria, whilst there are others who adopt the attitude that essential differences do not exist. Whilst not inclining us to either of these extremes, the results recorded above do seem to bring vaccinial immunity more into line with the more fully investigated antibacterial immunity.

The fact that the blood of normal rabbits is able to fix and destroy virus *in vitro* does not necessarily imply that normal animals possess a hæmatogenous resistance against virus invasion. The *in vitro* phenomena may depend entirely upon the fact that virus used experimentally is always in association with

particulate matter which inevitably attracts phagocytic cells. It is conceivable that if virus could be introduced in pure saline suspension without any trace of other particulate matter the blood-cells might remain entirely unconcerned.

The blood of immune rabbits has been shown to possess greater viricidal power than normal blood; this is most probably a specific effect following upon virus infection, for it must be remembered that the tissue proteins contained in the immunizing inocula were derived from the same species and hence, presumably, devoid of antigenic effect.

There is much evidence, but not yet absolute proof, that the immune tissue cell in its action upon virus differs from the normal tissue cell—in other words, that there is a cellular immunity against vaccinia virus as well as a humoral immunity.

The immune serum constituents appear to possess a function other than that of virus neutralization. In some way, not yet understood, they increase the viricidal action of tissue-cells. In one experiment, not reported here, it was found that heating the immune serum had little effect upon this function. There are many possibilities as to its nature; perhaps the most attractive is an opsonic action on the virus. It is hoped, in subsequent work, to gain further information on this point.

SUMMARY.

(1) The white cells of normal blood were shown to take up vaccinia virus *in vitro*.

(2) Normal whole blood possesses some degree of viricidal power which is largely dependent upon a thermolabile constituent of the serum.

(3) Different anticoagulants have different effects upon the viricidal activity of the blood. Heparin is shown to be a suitable anticoagulant for *in vitro* experiments of the type recorded.

(4) The blood, spleen and testis of an immune rabbit show far greater viricidal power than those of a normal rabbit.

(5) Evidence is put forward which suggests that the increase of viricidal power by immunization depends upon—

(*a*) Alterations of the tissue-cells themselves—cellular immunity.

(*b*) An opsonic or perhaps a stimulin action of some of the serum constituents—humoral immunity.

We wish to record our indebtedness to Dr. C. H. Andrewes for much constructive criticism throughout the course of the research; and to Dr. E. H. J. Schuster for the schematic representation produced on p. 108.

REFERENCES.

Andrewes, C. H.—(1928) *J. Path. Bact.*, **31**, 671.
Douglas, S. R.—(1916) *Proc. Roy. Soc.*, B, **89**, 335.
Idem, Smith, W., and Price, L. R. W.—(1929) *J. Path. Bact.*, **32**, 99.
Fleming, A.—(1926) *Brit. J. Exp. Path.*, **7**, 281.
Smith, W.—(1929) *Ibid.*, **10**, 93.
Todd, C.—(1928) *Ibid.*, **9**, 244.
Walsh, V. G., and Harmsworth, D. (1927)— *Ibid.*, **8**, 135.

TYPHOID AGGLUTINATION AFTER ORAL IMMUNIZATION.

ADRIANUS PIJPER AND HELEN DAU.

From the Authors' private laboratory, Pretoria, South Africa.

Forwarded for publication February 12th, 1930.

WE set out to answer the following questions:

1. Does oral immunization against typhoid fever by Besredka's method produce agglutinins in man?
2. If produced, are these agglutinins large-flaking, small-flaking, or both?
3. If produced, are these agglutinins ever present in such quantities as to interfere with proper serological diagnosis?

These points are of special interest in South Africa, because the oral method of typhoid immunization is being used here on an ever-increasing scale, as detailed by Cluver (1929). In Pretoria alone, with a population of 70,000, thousands of prophylactic doses have been distributed during the last five years (Boyd, 1928). We call oral immunization by Besredka's method the ingestion on 3 consecutive days on an empty stomach of one pill containing dried bile, followed within 20 minutes to half an hour by a pill containing about 40,000 million dried typhoid bacilli, killed by heat. As there is no paratyphoid in Pretoria, all efforts can be concentrated on typhoid. Subcutaneous inoculation is rare amongst the civil population.

In animals an increase in agglutinins after oral immunization was observed by Otten and Kirschner (1926) for dysentery, by Burke and La Verne Barnes (1926) for typhoid, by Engelhardt and Kay (1927) for cholera, by Reiter (1926) for typhoid and dysentery, and no increase by Kosmodemiansky (1928) for paratyphoid B. In man an increase was found by Gloukhoff, Sokolowa and Goremykina (1925) for cholera, a very small or no increase by Zoboli (1929), by Rosa (1928), and by Germino (1928) for typhoid, and no increase by Engelhardt and Kay (1927) for cholera. The type of agglutinins is not mentioned by these authors. Hoffstadt, Thompson and Martin (1929) found an increase in agglutinins in students after oral typhoid immunization, but here again the type of agglutinin was not studied. Besredka himself (1925, 1927) denied the regular occurrence and the importance of serum antibodies after oral immunization.

We have often found agglutinins after oral typhoid immunization in chance observations. This suggested the desirability of recording the agglutination titre of several persons over a prolonged period after immunization, determined at weekly intervals. The graphs of nine such persons are given.

Cases I to VII are female native (Bantu) patients of the Pretoria Mental Hospital. There had been no case of typhoid fever in this department for

over two years when we started. Cases VIII and IX are white volunteers. Case IX had typhoid fever in 1913; of all the others nothing is known in this respect. Case 4 got unexpectedly discharged from the hospital.

In all these cases a preliminary examination of the blood, 1 week before immunization, showed the complete absence of agglutinins in a dilution of 1 : 100 and over. The arrow indicates the day on which immunization was started. Thereafter titrations were done on the same day of the week, at the same hour, for a period of 20 weeks, in dilutions from 1 : 100 to 1 : 3200. The suspensions used were: (1) A live suspension of *B. typhosus* H 901 (Weil and Felix). (2) A live suspension of *B. typhosus* O 901 (Felix and Olitzki). (3) An alcoholic suspension of typhoid bacilli (from the Oxford Standards Laboratory). To these three suspensions refer respectively the thin drawn line, the broken line, and the line of dashes and dots.

In all cases the last definite trace was taken as the end-point. Readings were taken after 2 hours in the incubator and 22 hours on the bench, except in the case of the alcoholic suspension, which was kept at 53° C. in a water-bath. All serums were used unheated so as not to spoil the O-agglutinins (Felix and Olitzki, 1929), and no antiseptics were added, for the same reason (Felix and Olitzki, 1928).

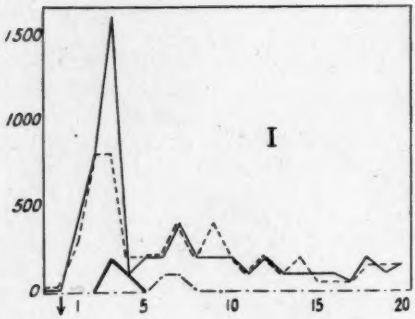
We have never found H-agglutinins in orally immunized persons. The graphs therefore refer to O-agglutinins only.

The reactions of the nine persons are very similar in type. They always showed agglutinins after 1 week, reached their maximum after 3 to 4 weeks, and thereafter the graphs became irregular, agglutinins sometimes being absent the one week and present again the next week. In most cases they tend to disappear after 20 weeks or so, some cases (VII and VIII) coming down much quicker than others.

The fact that O-agglutinins were found exclusively requires special attention. The diagnostic importance of this type of agglutinins was established by Felix (1924, 1929). In South Africa, realizing that the "ordinary Widal," which practically means H-agglutination, was unsatisfactory as a diagnostic test, we have used complement-fixation in typhoid fever for many years (Pijper, 1923, and Pijper and Pullinger, 1928). Complement fixers and O-agglutinins are closely related if not identical. Recently we have returned to agglutination reactions for diagnostic purposes (Pijper, 1930), as we found that by using the very sensitive suspensions mentioned above, the specific antibodies can be demonstrated at least as well in the form of O-agglutinins as in the form of complement-fixers. Moreover the technique of agglutination is much simpler than that of complement-fixation. We wish to emphasize that the greatest possible number of positive serological reactions in typhoid fever can only be achieved by paying close attention to O-agglutination. We feel sure that this does not only apply to South Africa.

For these reasons the observation that orally immunized persons possess O-agglutinins required further investigation on quantitative lines.

The thin lines in the figures refer to the reading of traces. These would never be regarded as diagnostic. We have, as the result of our experience, come to the conclusion that only marked agglutination with these particular suspensions in a dilution of 1 : 100 of the patient's serum can be regarded as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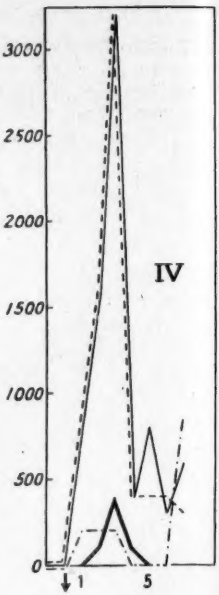
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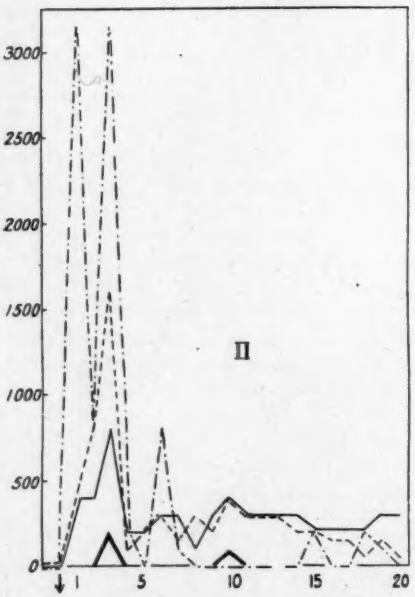
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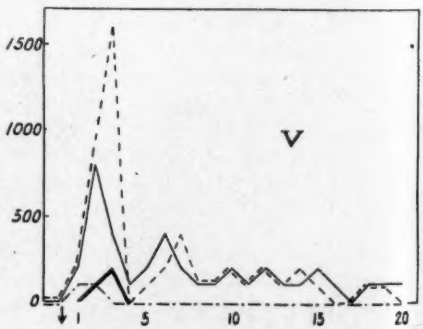
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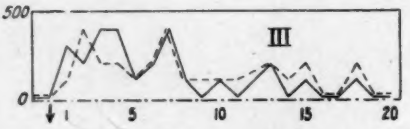
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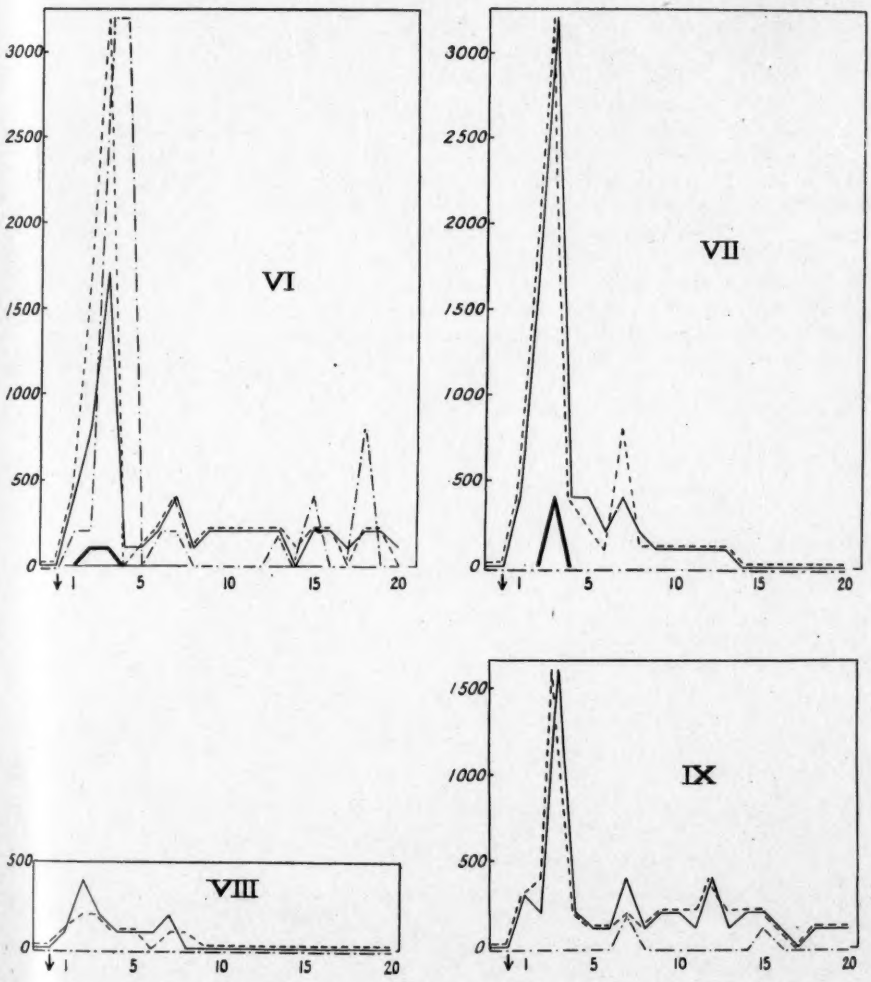
diagnostic. We have never seen "normal" agglutinins reach this titre, although others differ (Felix, 1929; Gardner, 1929). The thick lines refer to what we would regard as diagnostic quantities of agglutination.



It is seen that such high titres never occurred—at least not during the 20 weeks of observation—in Cases III, VIII and IX. The other cases showed diagnostic quantities of agglutination as a rule in the third week after immunization, but sometimes also in the second and fourth week, and occasionally in

a dilution of over a hundred. Case II was the only case that showed them again in a later week.

It must be concluded that O-agglutination as such cannot be relied upon as a diagnostic measure round-about the third week after oral immunization, and that in a few cases caution has to be exercised even at a later date. This is unfortunate, because 3 or 4 weeks after oral immunization is often just the time when people become typhoid-suspects, the immunization having been undertaken on account of exposure to existing cases in the neighbourhood.

Felix (1929) has shown that normal and immune O-agglutinins fluctuate under the stimulus of typhoid infection, but not of other infectious diseases. Repeated examination, therefore, would be a sure way to overcome the difficulty indicated. In practice, however, we have not yet found it necessary to have recourse to this. In five typhoid suspects who had been orally immunized 2 to 3 weeks previously, we found such low titres that they could safely be ascribed to the immunization—a course that was fully justified by the further events. In four similar cases who had really contracted typhoid, as evidenced by positive blood-cultures, we found such marked O-agglutination that we thought a positive diagnosis justified. One of these in addition showed H-agglutinins. The fact, however, remains that if the laboratory is not informed that oral immunization has recently taken place, a certain though probably very small number of incorrect positive diagnoses will be made.

There is one other point to which we wish to call attention. From the graphs it is evident that the titres for *B. typhosus* H 901 and *B. typhosus* O 901 run a fairly parallel course. This is at it should be, as both indicate the amount of O-agglutinins. The titre for the alcoholic suspension, however, is seen to take an entirely independent course. Cases III, VII and VIII never showed any agglutination with the alcoholic suspension at all. In Cases I, V and IX agglutination with alcoholic suspension always was very poor. That this was not due just to lack of sensitiveness of this suspension is quite evident from Cases II and VI, where quite high titres are reached with the alcoholic suspension, quite independently of what occurred with the other two suspensions. A similar phenomenon has repeatedly been observed by us in patients' serums and also in rabbits' immune serums. We have fairly often failed to find agreement between O-agglutination with alcoholic suspensions and live suspensions. We shall return to this question at a later date.

SUMMARY.

Oral immunization against typhoid fever by Besredka's method in man produces agglutinins. These are of the O-variety only. The highest titre is reached after about 3 weeks. Traces can be found after 20 weeks. From 3 to 5 weeks after oral immunization the titre may be so high as to interfere with proper serological diagnosis, if one, as so often is the case, has to rely on O-agglutination solely. In quite recently immunized persons a serological diagnosis of typhoid fever can still be made with confidence if the serum shows a much higher titre than in ordinary cases. Other febrile diseases apparently do not raise the titre.

We wish to thank the Superintendent of the Pretoria Mental Hospital, Dr. F. D. Crosthwaite, for permission to make these observations, and the nursing staff of the native female department for their co-operation.

REFERENCES.

BESREDKA, A.—(1925) 'Immunisation Locale,' Paris (Masson & Cie).—(1927) *Bull. Inst. Pasteur*, **25**, 529.
BOYD, J. J.—(1928) *Brit. Med. J.*, **1**, 159.
BURKE, V., AND LA VERNE BARNES.—(1926) *J. Infect. Dis.*, **39**, 67.
CLUVER, E.—(1929) *Lancet*, **1**, 1302.
ENGELHARDT, W. E., AND KAY, J. C.—(1927) *Z. Hyg.*, **107**, 663.
FELIX, A.—(1924[1]) *Z. ImmunForsch.*, **39**, 127.—(1924[2]) *J. Immunol.*, **9**, 115.—(1929) *J. Hyg.*, **28**, 418.
Idem AND OLITZKI, L.—(1928) *Ibid.*, **28**, 55.—(1929) *Brit. J. Exp. Path.*, **10**, 26.
GARDNER, A. D.—(1929) *J. Hyg.*, **28**, 376.
GERMINO, A.—(1927) *Policlin.* (Sez. Prat.), **46**, 1672.
GLOUKHOFF, SOKOLOWA AND GOREMYKINA.—(1925) *Hyg. Epidémiol.*, **4**, 63.
HOFFSTADT, R. E., THOMPSON, R. L., AND MARTIN, C. L.—(1929) *Amer. J. Hyg.*, **9**, 1.
KOSMODEMIANSKY, W. N.—(1928) *C.R. Soc. Biol.*, **99**, 1916.
OTTEN, L., AND KIRSCHNER, L.—(1926) *Z. Hyg.*, **107**, 314.
PIJPER, A.—(1923) *S. Afr. Med. Rec.*, February 10th and 24th.—(1930) *J. Hyg.* (in the press).
Idem AND PULLINGER, B. D.—(1928) *Brit. Med. J.*, **1**, 587.
REITER, H.—(1926) *Deuts. med. Wschr.*, 946.
ROSA, F.—(1928) Abstract in *Zbl. Bakt.*, Ref., **95**, 256.
ZOBOLI, C.—(1928) *Policlin.* (Sez. med.), **12**, 647.

THE EXCRETION OF INSULIN IN URINE.

R. D. LAWRENCE,* KATE MADDERS, AND H. R. MILLAR.

From the Biochemical Department, King's College Hospital.

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IN a recent paper Partos (1929) claims to have extracted insulin from the urine of normal men and rabbits by a simple technique (see below). He found that the extract of urine passed two hours after carbohydrate food (or after 100 gm. glucose in man) reduced the blood-sugar when injected into rabbits, whereas similar extracts from the urine of starved animals and diabetic men

* Working on insulin for the Medical Research Council.

produced no hypoglycæmia. The active extracts could be inactivated by excessive heat and by trypsin, which are known to destroy the potency of insulin. From these experiments he reasonably concluded that insulin was excreted in normal urine after carbohydrate food. He also stated that the extracts inactivated by heat, etc., not only failed to produce hypoglycæmia, but were hyperglycæmic in their action. This hyperglycæmia he had ascribed in a previous paper (Partos and Svec, 1928) to the presence of creatinine.

These results seemed to promise a method of studying insulin action under different conditions of metabolism in diabetics receiving insulin—different conditions of hyper-, hypo- and normal glycæmia. Preliminary experiments were undertaken to establish the technique on a quantitative basis if possible. Healthy young adults were given 50–100 gm. of glucose by mouth and the urine voided two hours later was treated as suggested by Partos, but no evidence of insulin action has been obtained with such extracts on rabbits.

The following method of urine extraction was used by Partos and followed exactly by us in nine experiments:

30 c.c. of urine were evaporated on a water-bath at 58° C. The residue was extracted in 10 c.c. 80% alcohol in a water-bath at 40° C. for 15 minutes, filtered, and the filtrate evaporated at 40° C. The residue was extracted with 10 c.c. of ether for 10 minutes, the ether decanted and the last traces removed by warmth at 40° C. The residue was dissolved in 5 c.c. distilled water (its reaction was acid), filtered and injected subcutaneously into a rabbit.

Slight modifications of this technique also employed by us were:

(*a*) The omission of an extraction with ether.

(*b*) The use of 70% instead of 80% alcohol, as it was suggested by Dr. Dudley that this might extract more insulin.

The results of our experiments need not be given in detail, but are grouped under three headings. They were performed on two rabbits whose usual reaction to 1 clinical unit of insulin was constant and well known—a fall of blood-sugar concentration of 40–60 mgm. The blood-sugar estimations were carried out in duplicate by Maclean's method and the effect on the blood observed every ½ hour for 2 and sometimes 3 hours.

SUMMARY.

1. Nine experiments with "active" extracts failed to produce any hypoglycæmia.

2. Two injections of extracts in which the "insulin" was inactivated by heat failed to cause the hyperglycæmia reported by Partos.

3. Two intravenous injections of 0·01 gm. pure creatinine also failed to cause any hyperglycæmia.

REFERENCES.

PARTOS, A.—(1929) *Arch. ges. Physiol.*, **221**, 562.
Idem AND SVEC.—(1928) *Ibid.*, **219**, 481.

EXPERIMENTS ON THE CULTIVATION OF VACCINIA VIRUS.

H. B. MAITLAND AND A. W. LAING.

From the Department of Bacteriology and Preventive Medicine, University of Manchester.

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THE experiments recorded here are an extension of the work of Maitland and Maitland (1928) on the cultivation of vaccinia virus in a medium consisting of minced hen's kidney serum and Tyrode's solution, work which has been confirmed by Rivers, Haagen and Muckenfuss (1929), and Eagles and McClean (1929). Muckenfuss and Rivers (1930) have also substituted rabbit for hen material. The method has been employed by Andrewes (1929) for the cultivation of virus III. The experiments of Maitland and Maitland appeared to indicate that actively growing tissue was not an essential condition for the growth of virus. That the tissue in this medium, without virus, maintained for about five days its ability to grow when transplanted to plasma clot has been indicated by Rivers, Haagen and Muckenfuss (1929).

In the experiments to be described the virus has been carried through 21 successive cultures, and some of the conditions affecting its growth have been investigated.

TECHNIQUE.

The medium.—Rabbits have been used throughout. Blood, taken by cardiac puncture under ether anæsthesia, has been defibrinated and the fresh serum used. The tissues have come from the same animal. Cultures were put up as follows: About 0·33 c.c. of freshly-minced tissue was put into a wide tube. The inoculum has sometimes been diluted with Tyrode's solution so that the required amount was contained in 1 c.c.; sometimes it has been convenient to add it without dilution (0·1 c.c.). In the latter case 1 c.c. of Tyrode's solution was first added to the tissue and mixed well with it. Prolonged contact between tissue and virus prior to incubation has been shown by comparative experiments not to be necessary. The precaution has, however, been taken thoroughly to mix the inoculum and tissue by gentle shaking. 3 c.c. of serum, and Tyrode's solution to make the volume up to 10 c.c. were then added, the serum being added last. The mixture was distributed into Carrel flasks (5 cm.), 2 c.c. to each flask. Incubation was at 37° to 38° C.

Titration.—The contents of a flask were ground with sand and centrifugalized. The supernatant fluid was diluted, with M/50 phosphate solution,

pH 7·6, in multiples of five; 0·2 c.c. was inoculated intradermally into the shaved back of adult rabbits.

Storage.—The centrifuge tubes containing sediment and supernatant fluid, plugged with cotton, were put in glass jars fitted with a ground-glass stopper and stored at ± 5° C.

Bacterial contamination has been controlled at each step by aërobic culture in Hartley broth.

CULTIVATION IN KIDNEY AND TESTIS.

In Table I the course of the virus through 21 cultures (18 with kidney and 3 with testis) is shown. The inoculum for the first culture was the supernatant fluid from rabbit testis which had been infected with commercial vaccine lymph, ground with sand and centrifugalized. It was prepared for the earlier experiments of Maitland and Maitland (1928); it contained 50% glycerol, and had been stored at ± 5° C. for 115 days; 17 days before it was used for these cultures its infective titre was 1 in 1000.

The amount of growth in each set of cultures is indicated by comparing columns 7 and 8 with the results of titrating cultures after varying periods of incubation. The figures in column 7 represent the potency of the virus in the cultures at the time they were set up, expressed as its infective titre. They were calculated from the titre of the virus used as inoculum and the total dilution made in preparing the cultures, and do not allow for deterioration of the virus during storage. In some of the later experiments one flask of each set of cultures was titrated immediately after they were set up. There is evidence that the virus increased from 10 to 1000 times or more in almost every set of cultures. The potency of the virus in flasks incubated for 4 to 7 days was, as a rule, greater than after 2 to 3 days' incubation. Comparatively little virus served to initiate growth (cultures 2, 3 and 8). Virus has been subcultured after storage for as long as 191 and 128 days (cultures 7A and 9B), and frequently after storage for 2 to 4 weeks. It seemed that occasionally the virus had not increased equally in all the flasks of one set, a 7-day culture being weaker than a 4- or 5-days' culture (6A, 7A, 9A, 9B, 11B, 20). This irregularity represented, however, a difference of only one step in the series of dilutions made for titration. The highest titre obtained for each set of cultures varied from 1 in 625 to 1 in 390,000; of 31 sets a titre of at least 1 in 15,625 was obtained in 22. During the 21 successive cultures the virus has increased approximately $4{\cdot}38 \times 10^{44}$ times. The lesions in the skin of rabbits caused by the culture-virus have continued to be typical and have not become less intense.

Failures.—The cultures recorded in this paper were free from bacterial contamination. Contamination has not been a source of great difficulty, but some sets have been discarded for this reason. In some contaminated flasks the virus increased as usual, but sometimes contamination was associated with absence of growth of virus. At one period several successive failures were traced to irregular rises in temperature of the incubator to 40° C. or over. When this was corrected no further difficulty was experienced. Of the last 14 sets of cultures the virus multiplied in 13 which were free from bacteria.

The exception was contaminated, and the virus did not grow. It has also been thought desirable to keep the air in the incubator moist.

The association of virus with the tissue of the medium.—More virus was obtained from cultures when they were ground with sand and centrifugalized than when they were centrifugalized only; comparative titrations were made of the supernatant fluid in three instances. The results were as follows (the ground cultures are stated first): 2-day cultures, 1 in 15,625, 1 in 625; 5-day cultures, 1 in 3125, 1 in 625; 7-day cultures, 1 in 15,625, 1 in 625.

COMPARISON OF KIDNEY AND TESTIS.

Four cultures have been made, using testis as tissue for the medium. In each instance a comparison was made with cultures containing kidney. The results are summarized in Table II. The virus grew about equally with testis or kidney. In these experiments (except the first) the inoculum had come from a testis-culture. Growth of virus in association with one tissue did not prevent its growth when subcultured to the other. During three subcultures in testis (Table I) the virus increased approximately $6{\cdot}25 \times 10^7$ times. It was noted that the lesions following the injection of testis-cultures were usually larger, more severe, and sometimes appeared earlier than those caused by kidney-cultures (*cf.* Duran-Reynals, 1929). This phenomenon has been investigated by titrating Government lymph, diluted in phosphate, and diluted in phosphate containing 50% of testis extract. Three kinds of extract were tested: (*a*) the supernatant fluid obtained by grinding a testis with sand and 2 c.c. of M/50 phosphate pH 7·6; (*b*) the fluid from flasks of medium containing testis which were prepared as for cultures, but without inoculum, and incubated for 3 days; (*c*) the same as (*b*), but incubated for 7 days. It was desired to determine whether the testis extract merely augmented the lesions, or whether it affected the infective titre of the virus. The results are summarized in Table III. It seems safe to assume that the titre of the virus was not increased as a result of inoculating testis extract with it. The extract of fresh testis, which was also more concentrated than the other extracts, had the most marked effect on the lesions. The supernatant fluid from medium incubated for 3 days was more active than that obtained after 7 days' incubation.

THE BEHAVIOUR OF THE VIRUS IN A MEDIUM CONTAINING SPLEEN OR LIVER

Strictly comparable experiments in which spleen or liver has been substituted for kidney in the medium have indicated that the virus did not multiply in association with these tissues. The details are summarized in Table IV.

THE BEHAVIOUR OF THE VIRUS IN ANAËROBIC CULTURE.

Cultures containing kidney were incubated aërobically and in an anaërobic jar (Table V). The virus did not multiply under anaërobic conditions.

TABLE I.—*Showing the Details of 21 Passages of Virus in Culture.*

No. of culture.	Tissue in medium.	Source of inoculum (culture number).	Titre of inoculum before storage.	Age of inoculum.	Final dilution of inoculum in the culture.	Estimated titre of virus in the culture when set up.	Titre of cultures.					
							Before incubation.	After 2 days at 37° C.	After 3 days at 37° C.	After 4 days at 37° C.	After 5 days at 37° C.	After 7 days at 37° C.
1	Kidney	Rabbit testis	1/1000	115 days	1/15	1/60	..	>1/625 1/25	..	>1/625 1/25	..	*Nil*
2	,,	No. 1	>1/625	7 ,,	1/75	>1/8	..	..	1/625 1/625	..	1/625 1/125	1/625 1/625
3	,,	No. 2	1/625	5 ,,	1/100	1/6	..	..	..	..	1/3125 1/3125	>1/15,625 >1/15,625
4	,,	No. 3	>1/15,625	7 ,,	1/100	>1/150	..	>1/15,625	..	..	1/3125	>1/15,625
5	,,	No. 4	>1/15,625	5 ,,	1/100	>1/150	..	1/78,125 1/78,125	..	>1/78,125 >1/78,125	..	>1/78,125 >1/78,125
6	,,	No. 5	>1/78,125	26 ,,	1/100	>1/780	*1/125	1/125	..	..	1/78,125	1/78,125
6A	,,	No. 5	>1/78,125	26 ,,	1/1000	>1/78	*1/125	<1/125	..	..	1/78,125	1/15,625
6B	,,	No. 5	1/78,125	54 ,,	1/100	1/780	..	1/125	..	>1/3125	..	1/15,625
7	,,	No. 6B	1/15,625	16 ,,	1/100	1/150	..	1/125	..	..	..	1/3125
7A	,,	No. 6	1/78,125	191 ,,	1/100	1/780	..	1/625	..	..	1/3125	1/625
7B	,,	No. 6	1/78,125	9 ,,	1/100	1/780	..	1/625	..	..	*Nil*	*Nil*
8	,,	No. 7B	1/625	10 ,,	1/100	1/6	..	1/125	..	1/3125	..	1/3125
8A	,,	No. 7	1/3125	5 ,,	1/100	1/30	..	1/125	..	1/3125	..	1/3125
8B	,,	No. 7A	1/3125	7 ,,	1/100	1/30	..	1/125	..	>1/15,625	..	>1/15,625
9	,,	No. 8	1/3125	16 ,,	1/100	1/30	..	1/125	..	..	..	1/3125
9A	,,	No. 8A	1/3125	31 ,,	1/100	1/30	..	1/125	..	1/15,625	..	1/3125
9B	,,	No. 8A	1/3125	128 ,,	1/100	1/30	..	1/125	..	..	1/15,625	1/3125
10	,,	No. 9A	1/15,625	7 ,,	1/100	1/150	..	..	1/25	..	1/3125	>1/15,625
10A	,,	No. 9B	1/15,625	7 ,,	1/100	1/150	..	1/125	..	1/3125	..	>1/15,625
11	,,	No. 10	1/3125	7 ,,	1/100	1/30	..	1/125	..	1/3125	..	..
11A	,,	No. 10A	>1/15,625	4 ,,	1/100	>1/150	..	..	1/125	..	1/3125	1/625
12	,,	No. 11A	1/3125	26 ,,	1/100	1/30	..	1/625	..	1/625	..	>1/15,625
13	,,	No. 12	>1/15,625	4 ,,	1/100	>1/150	..	..	1/15,625	..	..	1/15,625
14	,,	No. 13	1/15,625	5 ,,	1/100	1/150	..	1/3125	..	..	>1/15,625	1/15,625
15	,,	No. 14	1/15,625	5 ,,	1/100	1/150	..	1/625	..	>1/15,625	..	>1/15,625
16	,,	No. 15	>1/15,625	7 ,,	1/100	>1/150	1/625	..	1/625	..	1/3125	1/15,625
17	,,	No. 16	1/15,625	5 ,,	1/100	1/150	1/3125	1/15,625	..	..	1/3125	>1/390,625
18	,,	No. 17	>1/390,625	16 ,,	1/100	>1/3900	1/3125	..	1/3125	..	1/15,625	1/15,625
19	,,	No. 18	1/15,625	28 ,,	1/100	1/150	1/625	1/3125	..	..	1/15,625	? 1/1,950,000
19A	Testis	No. 18	1/15,625	28 ,,	1/100	1/150	1/625	>1/15,625	..	..	1/15,625	? 1/1,950,000
20	,,	No. 19A	? 1/1,950,000	5 ,,	1/1000	? 1/1900	1/25	1/3125	..	1/78,125	..	1/15,625
21	,,	No. 20	1/78,125	7 ,,	1/100	.1/780	1/625	..	1/15,625	..	1/78,125	1/390,625

> = Not an end-point. * = After storage for 2 days at 5° C. Each figure represents the titration of one flask.

TABLE II.—*Comparison of Kidney and Testis.*

Tissue in medium.	Titre of cultures.					
	Before incubation.	After 2 days at 37° C.	After 3 days at 37° C.	After 4 days at 37° C.	After 5 days at 37° C.	After 7 days at 37° C.
Kidney . .	1/3125	..	1/3125	..	1/15,625	1/15,625
Testis . .	1/3125	..	1/3125	..	1/15,625	1/15,625
Kidney . .	1/625	1/3125	..	..	1/15,625	? 1/1,950,000
Testis . .	1/625	>1/15,625	..	..	1/15,625	? 1/1,950,000
Kidney . .	1/25	1/25	..	1/5	..	1/15,625
Testis . .	1/25	1/3125	..	1/78,125	..	1/15,625
Kidney . .	1/625	..	1/625	..	1/15,625	1/390,000
Testis . .	1/625	..	1/15,625	..	1/78,125	1/390,000

Each figure represents one flask. > = Not an end-point.

TABLE III.—*Effect of Testis Extract on Vaccinial Lesions in the Skin of Rabbits.*

Dilutions of Government lymph.	Size of 5-day lesions in rabbits, in millimetres.					
	Diluted in phosphate.	Diluted in extract A.	Diluted in phosphate.	Diluted in extract B.	Diluted in phosphate.	Diluted in extract C.
1/100,000	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*
1/10,000	Pap.	20 × 13	9 × 7	16 × 11	*Nil*	Pap.
1/1000	8 × 8	27 × 19 (N.A. 6×4)	11 × 9	30 × 22	10 × 7	16 × 14
1/100	9 × 9	32 × 25 (N.A. 23 × 14)	15 × 14	40 × 26	13 × 13	25 × 20

Pap. = Papule. N.A. = Necrotic area.

TABLE IV.—*Comparison of Kidney, Liver and Spleen.*

Tissue in medium.	Titre of cultures.					
	Before incubation.	After 2 days at 37° C.	After 3 days at 37° C.	After 4 days at 37° C.	After 5 days at 37° C.	After 7 days at 37° C.
Kidney . .	1/150*	1/3125	..	..	>1/15,625	1/15,625
Spleen . .	1/150	1/125	..	..	1/5	*Nil*
Kidney . .	1/150*	1/625	..	>1/15,625	..	1/15,625
Spleen . .	1/150	1/5	..	*Nil*	..	*Nil*
Kidney . .	1/625	..	1/625	..	1/3125	1/15,625
Liver . .	1/625	..	*Nil*	..	*Nil*	*Nil*
Kidney . .	1/3125	1/15,625	..	..	1/3125	> 1/390,000
Liver . .	1/3125	1/625	..	..	1/5	*Nil*

Each figure represents the titration of one flask.
> = Not an end-point. * = Estimated titre.

TABLE V.—*Comparison of Aërobic and Anaërobic Cultures in Kidney.*

—	Titre of cultures.					
	Before incubation (estimated).	After 2 days at 37° C.	After 3 days at 37° C.	After 4 days at 37° C.	After 5 days at 37° C.	After 7 days at 37° C.
Aërobic .	>1/150	..	1/125	..	1/3125	1/625
Anaërobic .	>1/150	..	*Nil*	..	*Nil*	*Nil*
Aërobic .	1/30	1/625	..	1/625	..	1/15,625
Anaërobic .	1/30	1/5	..	1/5	..	*Nil*

Each figure represents one flask. > = Not an end-point.

TABLE VI.—*Comparison of Cultures in Heated and Unheated Kidney.*

Degree of heating.	Titre of cultures.					
	Before incubation (estimated).	After 2 days at 37° C.	After 3 days at 37° C.	After 4 days at 37° C.	After 5 days at 37° C.	After 7 days at 37° C.
Unheated .	1/30	1/125	..	1/3125	..	1/3125
45° C. 20 min.	1/30	1/25	..	1/25	..	*Nil*
Unheated .	1/30	1/125	..	..	1/3125	..
45° C. 20 min.	1/30	1/5	..	..	*Nil*	..
Unheated .	1/30	1/125	..	1/15,625	..	1/3125
40° C. 20 min.	1/30	1/125	..	1/15,625	..	1/5
Unheated .	1/150	..	1/25	..	1/3125	>1/15,625
40° C. 20 min.	1/150	..	1/125	..	1/3125	1/625
Unheated .	>1/150	..	1/15,625	..	(c) 1/3125	1/15,625
40° C. 20 min.	>1/150	..	1/3125	..	1/15,625	1/25

Each figure represents one flask. > = Not an end-point. (c) = Bacterial contamination.

TABLE VII.—*Re-titration of Virus after Storage.*

Number of culture.	Original titre.	Duration of storage.	Titre after storage.
15	>1/15,625	17 days	1/3125
8B	>1/15,625	84 ,,	1/125
16	1/15,625	5 ,,	1/15,625
17	1/3125	7 ,,	1/625
5	1/78,125	358 ,,	1 3125
9	1/15,625	149 ,,	1/3125
17	>1/390,625	16 ,,	1 78,125
18	1/15,625	28 ,,	1/15,625
19A	? 1/1,950,000	5 ,,	1/15,625
20	1/78,125	7 ,,	1/78,125

> = Not an end-point. ? = Discussed in text.

CULTIVATION OF VIRUS IN MEDIUM CONTAINING HEATED TISSUE.

The growth of virus in medium containing fresh kidney has been compared with its behaviour in medium containing kidney heated for 20 minutes at 45°C. and at 40°C. (Table VI). Heating the kidney at 45°C. (mixed with Tyrode's solution in a thin glass tube in a water-bath) made it unsuitable for growth of virus. Heating at 40°C. did not alter its function for promoting growth of the virus during the first 4 or 5 days of incubation, but after 7 days' incubation the cultures with heated kidney contained much less virus than those with fresh kidney. It appeared that in the cultures with heated kidney the virus rapidly became non-infective between the 5th and 7th days of incubation. A possible explanation is that the preliminary heating caused a more rapid disappearance during incubation of the essential function or functions of the tissue which are concerned in promoting growth of the virus.

STORAGE OF CULTURE-VIRUS.

Only 10 samples of virus have been re-titrated after storage under routine conditions. The results are shown in Table VII. Two samples tested after storage for 358 and 149 days respectively were infective in a dilution of 1 in 3125. The others tested after shorter intervals were also infective.

TITRATION.

Adult chinchilla rabbits, fairly uniform in weight, have been used throughout, and there has been little evidence of variation in individual susceptibility. Comparative experiments have always been titrated on one animal. Each animal has not received more than 10 to 12 injections. The end-point for the 7-day flasks of cultures 19 and 19A is probably too high, and may have been due to unusual susceptibility of the rabbit, or to the localization of virus from the lesions caused by lower dilutions, *viâ* the blood, in areas inoculated with higher dilutions. The final results have, as a rule, been read on the 7th day. At this time generalized lesions in the skin were sometimes present, and it is possible that they may appear only in inoculated areas which up to this time have not shown lesions. It is thought, however, that this complication has been avoided by observing the daily progress of the lesions.

SEROLOGICAL REACTIONS.

Virus from the 16th culture was neutralized by antivaccinal serum prepared against dermal vaccine. This virus, diluted in M/50 phosphate, pH 7·6, was infective in a dilution of 1 in 3125; when diluted with 50% serum in phosphate and left at room temperature for ½ hour before injection, a dilution of 1 in 50 did not produce a lesion.

The serum of rabbits after recovery from infection with culture-virus fixed complement specifically, Government lymph being the antigen. The serum of a rabbit hyperimmunized with Government lymph fixed complement specifically when culture virus was the antigen. (The complement-fixation experiments have been made by Dr. E. StG. Gilmore.)

SUMMARY AND CONCLUSIONS.

Vaccinia virus appears to be capable of cultivation indefinitely in the medium described. It has been carried through 21 cultures; 19 culture passages have been made in medium containing kidney and 3 with testis.

In almost all sets of cultures the virus increased from 10 to 1000 times or more. In 22 of 31 sets the titre of the virus was at least 1 in 15,625. Cultures contained more virus after incubation for 4 to 7 days than after 2 to 3 days' incubation. The amount of growth of virus may not always have been equal in all the flasks of one set. Comparatively little virus served to initiate growth. During cultivation the virus has maintained its ability to produce typical lesions in the skin of rabbits.

Virus has been subcultured after storage for 191 and 128 days. Samples have been infective when tested after storage for from 5 to 358 days.

In view of the regularity with which cultures have been obtained, the potency of culture-virus and the maintenance of its infectivity during storage, its freedom from bacterial contamination, and the small cost entailed, this or a similar method of culture suggests itself as being suitable for the production of "vaccine" on a larger scale.

The virus has multiplied about equally in medium containing kidney or testis. Extracts of testis have been noted to augment the lesions produced by the virus in the skin of rabbits. The virus did not multiply in medium containing spleen or liver, nor in association with kidney under anaërobic conditions.

Kidney which had been heated at 45° C. for 20 minutes before it was incorporated in the medium did not promote the growth of virus. Kidney heated at 40° C. for 20 minutes promoted the growth of virus, as well as unheated kidney, during the first 4 or 5 days of incubation. Between the 5th and 7th days the amount of virus in the cultures with heated kidney rapidly decreased.

The culture virus was neutralized *in vitro* by antivaccinal serum prepared against dermal vaccine. Serum of rabbits which had been immunized with culture-virus fixed complement specifically when Government lymph was used as antigen; conversely, serum from rabbits immunized with Government lymph fixed complement when the antigen was culture-virus.

We wish to acknowledge the kindness of Dr. F. R. Blaxall in supplying Government lymph, and Prof. J. C. G. Ledingham, F.R.S., who provided antivaccinial serum.

REFERENCES.

ANDREWES, C. H.—(1929) *Brit. J. Exp. Path.*, **10**, 188.
DURAN-REYNALS, F.—(1929) *J. Exp. Med.*, **50**, 327.
EAGLES, G. H., AND MCLEAN, D.—(1929) *Brit. J. Exp. Path.*, **10**, 35.
MAITLAND, H. B., AND MAITLAND, M. C.—(1928) *Lancet*, **2**, 596.
MUCKENFUSS, R. S., AND RIVERS, T. M.—(1930) *J. Exp. Med.*, **51**, 149.
RIVERS, T. M., HAAGEN, E., AND MUCKENFUSS, R. S.—(1929) *Ibid.*, **50**, 181.

ON THE OCCURRENCE OF INFLUENZA BACILLI IN THE MOUTHS OF NORMAL PEOPLE.

ALEXANDER FLEMING AND IAN H. MACLEAN.

From the Inoculation Department, St. Mary's Hospital, London.

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IN view of the importance which has been ascribed to bacilli of the influenza group it is obvious that reports of their occurrence in the normal man are worth recording. Many observations have been made on this subject, but with few exceptions these have referred only to the throat (tonsils and pharynx). The reports prior to 1918 can be neglected as the methods in use for the isolation of bacilli of this group were so inadequate that they need not be considered. A revolution in the culture methods for the influenza group of bacteria occurred in 1918, when blood, changed in various ways, was used in the medium, so that the bacilli grew luxuriantly instead of in the pin-point colonies which had so long been seen on blood-agar. This change of medium was originally introduced by Cohen and Fitzgerald (1910), who altered the blood by heat, but it had been completely forgotten, and was revived by Matthews (1918) (trypsin digest of blood), Levinthal (1918) (boiled and filtered blood), and Fleming (1919) (blood acidified and then neutralized).

Since the influenza pandemic of 1918–1918 many observations have been made on the occurrence of influenza bacilli in the throats of normal people. It is unnecessary to quote results other than those set out in Table I.

TABLE I.—*Typical Results Obtained by other Observers in the Isolation of Influenza Bacilli from Normal Individuals.*

Observer.	Date.	Source of culture.	Percentage of positive examinations.
Pritchett and Stillman	1919	Throat	30
		Saliva	25
Scott	1920	Post-nasal space	70
	1925	,,	50
Topley and Wilson	1925–1927	,,	43 to 80

It will be seen from these figures that in seasons when there is no influenza pandemic the proportion of normal people who are carriers of influenza bacilli may be very high (up to 80%), while Pritchett and Stillman's figures show that in 1918 there was a goodly proportion of healthy people who had these bacilli in their saliva.*

* Pritchett and Stillman's observations were made before there was a clear distinction between *B. influenzæ* and *B. parainfluenzæ*.

In July last Fleming (1929) described a filtrate of a culture of a penicillium which had the property of completely inhibiting the Gram-positive cocci and the diptheroid bacilli, while it had practically no inhibitory power on the members of the influenza group, and he showed that by the use of this filtrate (penicillin) it was easy to isolate influenza bacilli even when these were present amidst multitudes of pneumococci or streptococci.

In this communication we propose to set forth the results we have obtained by the use of penicillin in the isolation of bacilli of the influenza group from normal healthy individuals, and especially from the gums. These observations were made in January and February of 1930.

Some preliminary experiments had led us to believe that hæmoglobinophilic bacilli were not uncommon on the gums, but little or no work has been done in this connection. We originally set out to examine the gums of 100 normal people, and at the same time we examined specimens from the tonsils and post-nasal spaces for comparison. The first specimens were taken from 10 nurses and 10 students at St. Mary's Hospital, and 10 dental students from another institution. Swabs were taken from the tonsils, post-nasal spaces and gums, and from these swabs implants were made on plates of boiled blood-agar (chocolate agar). Then some 6 to 8 drops of penicillin were spread over half of each plate. The plates were incubated for 24 (or occasionally for 48) hours, and then suspected colonies were pricked off on to other plates of the came medium. These were cultured and examined morphologically and sulturally.

It is necessary here to state our definition of the influenza group of bacilli. They are Gram-negative non-motile bacilli of various length and appearance which grow profusely on boiled blood-agar, not at all on agar, and which on blood-agar grow in minute colonies except in proximity to colonies of some other bacterium such as staphylococcus.

Table II shows the results obtained from these 30 normal people.

TABLE II.—*Occurrence of Hæmoglobinophilic Bacilli in 30 Normal Individuals.*

Group.	Number.	Number of positive results from specimens from—		
		Tonsil.	Post-nasal space.	Gums.
Nurses	10	10	9	10
Students	10	10	9	10
Dental students	10	9	8	10
Total	30	29	26	30

The figures given in this table show that in practically every person bacilli of the influenza group were present in all the situations tested. In the one or two cases where the bacilli were not found we have no doubt that this was due to accident, as in every case where this occurred the plates were either too thickly or too thinly spread.

After isolation the cultures were streaked on blood-agar. Table III shows that most of the cultures were non-hæmolytic, but there was a certain proportion of hæmolytic strains from all sources.

TABLE III.—*Occurrence of Hæmolytic and Non-Hæmolytic Types.*

	Tonsil.		Post-nasal space.		Gums.	
	Lytic.	Non-lytic.	Lytic.	Non-lytic.	Lytic.	Non-lytic.
10 nurses	1	10	1	9	0	10
10 dental students	2	7	1	7	4	9
20	3	17	2	16	4	19

Cultures were also made in streaks on agar and on autoclaved blood-agar. It has been established that for the growth of *B. influenzæ* two factors are necessary—the X factor (hæmatin in these media), and the V factor which is thermolabile and which is elaborated by many bacteria, *e.g.* staphylococci. Staphylococci was therefore stroked on the plates across the streaks of the isolated bacilli. The agar plate therefore contained no X or V factors, except in the neighbourhood of the staphylococcal streaks where the V factor was present. The autoclaved blood-agar contained the X factor only, except near the staphylococcal streak, where X and V factors are both present.

On such plates *B. influenzæ* proper (which requires both X and V factors) will, on the autoclaved blood-agar plate, grow only in the neighbourhood of the staphylococcus streak, and will not grow on the agar plate even near the staphylococci. On the other hand, *B. para-influenzæ* will grow on both plates near the staphylococcus streak.

Table IV shows that most of the cultures isolated were by their growth characteristics true influenza bacilli, although a number of para-influenza bacilli were present, and one bacillus was isolated which had the characters of *B. hæmoglobinophilus canis*.

TABLE IV.—*Occurrence of* B. influenzæ *and* B. para-influenzæ.

	Tonsil.		Post-nasal space.		Gums.	
	Inf.	Para.	Inf.	Para.	Inf.	Para.
10 nurses	10	4	9	1	10	4
10 dental students	9	0	8	0	9*	1

The results obtained with these first cases convinced us that members of the hæmoglobinophilic group were constant inhabitants of the gums of normal individuals, and so we considered it was waste of time to complete our programme of a longer series on these lines. We therefore next took a small series of 6 laboratory workers and examined their gums by a more thorough method, with the object of seeing what different varieties of hæmoglobinophilic bacteria were present. The examinations were carried out in the following manner: Swabs

* One culture in addition grew well on medium containing the X factor only, and did not grow on agar or agar + V factor. This corresponds to *B. hæmoglobinophilus canis*.

were taken from the gums, and these were stirred round in tubes of Fildes broth. One c.c. of this inoculated broth was then added to various quantities of penicillin to make the final concentration of penicillin 1 in 3, 6, 11, 21, 51 and 100. A control in which there was no penicillin was also used. These mixtures were incubated for 24 and 48 hours and a loopful of each was planted on boiled blood agar. From these plates suspected colonies were pricked off and examined. Generally speaking the control tubes contained large numbers of streptococci and sometimes diphtheroid bacilli, with smaller numbers of Gram-negative cocci and bacilli resembling influenza bacilli. The lesser concentration of penicillin contained many Gram-negative cocci and a few influenza bacilli with occasional Gram-positive cocci. The higher concentrations of penicillin contained many influenza-like bacilli, sometimes pure and sometimes with Gram-negative cocci. Of the cultures obtained those which morphologically resembled *B. influenzæ* were streaked on blood-agar, on agar and on autoclaved blood-agar and a cross streak of staphylococcus was made on each plate.

TABLE V.—*Occurrence of Hæmoglobinophilic Bacteria in the Gums of 6 Laboratory Workers.*

Hæmoglobinophilic bacilli present	6
Non-hæmolytic types present	6
Hæmolytic types present	4
Types requiring X and V factors present . . .	6
Types requiring V factor only present . . .	6

Table V shows that non-hæmolytic types of *B. influenzæ* and *B. para-influenzæ* are constant inhabitants of the gums, while the hæmolytic types were present in a large percentage.

As regards morphology, the bacilli were very variable. They differed in length from something under 1μ to long threads. Some of these threads were very irregular and swollen in places—a feature which has frequently been noted. These long irregular forms are known in our laboratory parlance as "sea serpents," and are so referred to in Table VI, which shows the occurrence of the different morphological types in the gums of the people tested.

TABLE VI.—*Morphology of Hæmoglobinophilic Bacilli Isolated from the Gums of 26 Normal Individuals.*

Coccal.	Short.	Medium.	Long.	"Sea serpents."
11	32	13	11	3

11 individuals	had 1	morphological	type.
17 ,,	,, 2	,,	types.
7 ,,	,, 3	,,	,,
1 individual	,, 4	,,	,,

It will be seen from the table that most of the people (normal individuals) had at least two types and some had three or four.

The cultural types were likewise extremely varied. Table VII gives an analysis of the varieties isolated from the gums of 5 laboratory workers, who were worked out in the greatest detail.

It will be seen from this table that the same mouth may contain *B. influenzæ* hæmolytic and non-hæmolytic with different cultural characters and para-influenza bacilli apparently quite different from each other.

One type of parainfluenza bacillus was constant in all these 6 laboratory workers. In its morphology it was a very fine short bacillus with longer elements and always clumpy. On boiled blood-agar it gave a large colony

TABLE VII.—*Showing some of the Characters of Different Hæmoglobinophilic Bacilli Isolated from 5 Laboratory Workers.*

No.	Colony on boiled blood-agar.	Morphology.	Hæmolysis.	Culture in Fildes broth.	Classification according to nutrient factors.
1	Large regular	Long	0	Rough	Influenza.
	Small „	Short	0	Smooth	„
	„ „	Long	0	Rough	„
	„ with flat irregular margin	Short	0	„	„
	Large irregular	Long	+	...	„
	„ wrinkled tough	Short	0	Rough	Para-influenza.
2	„ woolly	Medium	+	„	„
	„ wrinkled tough	Short	0	„	„
	Small regular	Medium	0	„	Influenza.
3	Large wrinkled tough	Short	0	„	Para-influenza.
	Medium regular	Medium	0	Smooth	Influenza.
	Small „	Short	0	„	„
	„ flat irregular	„	0	Rough	„
	Large regular	„	+	„	„
4	„ wrinkled tough	„	0	„	Para-influenza.
	Small flat irregular	„	0	„	Influenza.
	„ regular	Medium	0	Smooth	„
5	Large wrinkled tough	Short	0	Rough	Para-influenza.
	„ regular	Long	0	Smooth	„
	Small „	Short	+	Rough	„
	„ „	„ (pleomorphic)	+	Smooth	Influenza.

which was flattish and very wrinkled. When touched with a platinum wire the whole colony could be pushed over the plate, and it left on the medium a very distinct pattern of the colony. On blood-agar it grew as a tiny non-hæmolytic colony except in the neighbourhood of another bacterium, *e. g.* staphylococcus, where the growth was much more copious. On autoclaved blood-agar there was no growth except in proximity to the staphylococcus growth, where it showed the same rough wrinkled colony previously noted on boiled blood-agar. On agar there was no growth except near a staphylococcus colony, where a wrinkled colony developed. In Fildes broth the growth was very granular. All of 8 strains tested fermented glucose and saccharose, but not lactose, mannite or dulcite. No indol was produced. This microbe obviously liked hæmatin, as the growth was much more copious on boiled blood-agar than it was on agar plus staphylococcus, but as it survived at least 6 subcultures on a

medium containing the V factor only it must be classed as a parainfluenza bacillus.

Subsequent to our work on normal people we have cultivated by the same methods from the apices of four extracted teeth. From one of these no growth of any kind was obtained, the second showed a non-hæmolytic parainfluenza, the third an influenza bacillus, while the last gave cultures of both influenza and parainfluenza bacilli.

These teeth were extracted with every precaution to prevent contamination of the apices from the gum margin, but it is, of course, impossible to be quite certain that no contamination occurred. The presence of these bacilli in cultures from the apices makes it seem possible that they may play some part in the infective processes for which the teeth were extracted.

METHODS OF USING PENICILLIN FOR THE ISOLATION OF INFLUENZA BACILLI.

There are three methods in which this substance may be used. The simplest, which is sufficient for most routine work, is that which we employed in the first series of 30 cases cited in this paper. The infected material is spread on a plate of suitable medium, and then 4 to 8 drops of penicillin are placed on half the plate and gently spread over the surface of that half. This method has the great advantage that on a single plate there is one half which gives the complete culture, and the other half (on which the penicillin was spread) in which the Gram-positive cocci and some other organisms are completely inhibited.

A second method, which is more troublesome but very much more effective, is that which we used in our second series of 6 laboratory workers. Various dilutions of penicillin are made in a suitable medium (Fildes broth), and these are implanted with the infected material, incubated for 24 or 48 hours and plated out. The actual dilutions employed will, of course, depend on the strength of the penicillin, but we have not found an influenza bacillus which is inhibited by a concentration of penicillin 40 times greater than that necessary to completely inhibit staphylococcus. The penicillin, therefore, should be titrated with staphylococcus, and then dilutions should be made up 40 and more times stronger than that which inhibits the staphylococcus. As an example, if any sample of penicillin inhibits staphylococcus in a dilution of 1 in 400, the suitable dilutions to employ for the isolation of influenza bacilli would be 1 in 3, 1 in 6 and 1 in 10.*

The strongest concentration may inhibit growth altogether, but in a number of cases we have obtained in one or more of the strongest concentrations a pure culture of a hæmoglobinophilic bacillus when by ordinary plating we missed it completely.

The third method is to incorporate into a suitable solid medium penicillin in concentrations similar to those employed in the fluid medium in the last method. When infected material from the gums is spread on plates of medium made in this way we have sometimes obtained pure cultures of influenza bacilli on the plates containing the greatest concentrations of penicillin, whereas on

* For this purpose the penicillin should be neutralized, as when made it is strongly alkaline. This neutralization greatly enhances its keeping properties.

those with less than 1 in 10 penicillin the majority of the colonies which appeared were Gram-negative cocci.

DISCUSSION.

It has been shown that *B. influenzæ* is present on the gums of all normal people. Para-influenza bacilli were present also in all those in which the most thorough examination was made. On the tonsils and in the post-nasal space influenza bacilli could be isolated from practically every individual, and we have no doubt that in the few negative cases the failure to find them was simply due to faulty technique.

The bacilli isolated were most varied in their cultural and morphological characters, and in most cases differed grossly from the typical Pfeiffer's bacillus, which was universally found in the great influenza pandemic. They were, however, dependent on the same nutrient factors as the true Pfeiffer's bacillus, and, as the relation to these nutrient factors is at present the only means of classifying the hæmoglobinophilic bacilli, they have to be classed as *B. influenzæ*. It seems clear that the name *B. influenzæ* is applied to a number of different bacteria which have grossly different cultural and morphological characters, and which appear to vary widely in their pathogenicity. No one will deny a pathogenic *rôle* to the true Pfeiffer's bacillus, but it has yet to be established that these normally occurring variants are concerned with any infective process.

The para-influenza bacilli, also, which are apparently constantly present in the mouth, may or may not be pathogenic. They have generally been regarded as harmless, but Russell and Fildes (1928) have shown that they are capable of giving rise to endocarditis and septicæmia. These, like the influenza bacilli, are most varied in their characters, and it is hardly possible that they are one and the same organism.

We have in this paper indicated methods whereby members of this group of bacilli can be isolated even when present in small numbers, but a good method for their classification has yet to be evolved. It is evident that more work must be done to show whether the Pfeiffer's bacillus, which occurs almost universally in epidemic influenza, has any close relation to the varied types inhabiting the mouth. The fact that influenza bacilli were almost constantly present in cases of epidemic influenza had been frequently used as an argument for the causal relationship of the bacillus to the disease, but this is considerably weakened by the realization that it is present constantly in the normal mouth in a variety of forms almost comparable with the ubiquitous streptococcus.

SUMMARY.

1. By the use of penicillin hæmoglobinophilic bacilli have been isolated from the gums of all of 30 healthy nurses and students, and from the tonsils and post-nasal spaces of almost all of these.

2. The majority of the strains isolated were non-hæmolytic, but hæmolytic strains were found in all situations.

3. Para-influenza as well as influenza bacilli were isolated from all these situations. A bacillus agreeing in its (growth) requirements with *B. hæmoglobinophilus canis* was isolated from the gums of one individual.

4. From the gums of 6 laboratory workers, who were studied very carefully, influenza and para-influenza bacilli were isolated from all.

5. The hæmoglobinophilic bacteria isolated were very variable in their cultural and morphological characters.

6. A type of para-influenza bacillus which was constantly found in the gums is described.

7. From the apices of infected teeth hæmoglobinophilic bacilli were isolated in 3 cases out of 4.

8. The methods of using penicillin for the isolation of influenza bacilli are described.

REFERENCES.

COHEN, C., AND FITZGERALD, J. G.—(1910) *Zbl. Bakt.*, Abt. 1, Orig. **56**, 464.
FILDES, P.—(1920) *Brit. J. Exp. Path.*, **1**, 129.
FLEMING, A.—(1919) *Lancet*, 1, 138.—(1929) *Brit. J. Exp. Path.*, **10**, 226.
LEVINTHAL, W.—(1918) *Z. Hyg. InfektKr.*, **86**, 1.
MATTHEWS, J.—(1918) *Lancet*, **2**, 104.
PRITCHETT, I. W., AND STILLMAN, E. G.—(1919) *J. Exp. Med.*, **29**, 259.
RUSSELL, DOROTHY S., AND FILDES, P.—(1928) *J. Path. Bact.*, **31**, 651.
SCOTT, W. M.—(1929) Medical Research Council's 'System of Bacteriology,' **2**, 366. London (H.M. Stationery Office).
TOPLEY, W. W. C., AND WILSON, G. S.—(1929) 'The Principles of Bacteriology and Immunity,' **2**, 1896. London (Arnold).

THE ELECTRICAL CHARGE OF YELLOW-FEVER VIRUS.

E. HINDLE,
Beit Memorial Research Fellow in Tropical Medicine,
AND
G. M. FINDLAY.
From the Wellcome Bureau of Scientific Research, London.

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No satisfactory method of treating yellow fever is known, and therefore any experiments which may throw light on a rational form of therapy are of value.

Hitherto, apart from purely symptomatic treatment, the only method which seems to offer any possibility of success is the use of immune serum (Marchoux, Salimbeni and Simond, 1903). It seems likely that if given

sufficiently early serum therapy may arrest the course of the disease, but in practice it is rarely possible to initiate treatment in the earliest stages of infection.

Brown and Broom (1929) have recently put forward a theory which may advance the chemotherapeutic treatment of certain diseases. In the case of leptospira, they show that *in vitro* the negative electric charge of the organisms is reduced by immune serum in the presence of complement, and when this occurs the leptospira are more susceptible to a negatively-charged colloid. It therefore seemed of interest to determine the electrical charge of yellow-fever virus as a preliminary to any chemotherapeutic experiments, in view of the fact that certain viruses such as that of foot and mouth disease are positively charged under ordinary conditions (Olitzky and Boëz, 1927), whilst others such as herpes are negatively charged at pH 7·6 and 8·0, but positively charged at pH 7·2, 7·0 and 6·6 (Bedson and Bland, 1929).

The electrical charge of virus particles can be determined, as shown by Bedson and Bland (1929), by immersing strips of blotting-paper in suspensions containing virus. If negatively charged, relatively to the blotting-paper, the virus particles ascend the paper, being carried upwards by the water. By this means the electrical charges of vaccinia and herpes virus have been determined.

The method has here been applied to the virus of yellow fever, the technique employed being similar to that of Bedson and Bland. As, however, the monkey is the only animal which is definitely susceptible to the action of yellow-fever virus, and economy in the use of animals is therefore essential, no attempt has been made to determine the presence of virus in separate centimetre lengths. After allowing the fluid containing the virus to ascend the blotting-paper, the portions of paper corresponding to 3 to 7 cm. inclusive above the level of the liquid were removed and ground up in a mortar with sterile buffer solution, the emulsion being then inoculated intraperitoneally into monkeys. It is obvious that by this means no evidence of the amount of virus present in strips 3 to 7 is obtained, and from the nature of the disease such would be extremely difficult to determine, for variations in the incubation period seem to depend as much on the susceptibility of individual monkeys as on the amount of virus present (within limits) in the infecting dose. The buffers employed in the experiments have been: phthalate/NaOH for pH 4·2 and 5·2, and KH_2PO_4/K_2HPO_4, M/50 for pH 5·6, 6·0 and 7·0.

The source of virus consisted either of serum from a monkey suffering from yellow fever, or of liver from a similar animal. The liver was emulsified in the buffer solution and then centrifuged to throw down gross particles. Experiments were carried out with two strains of yellow-fever virus, an African and a Brazilian, and similar results obtained with both. Virus was found in the blotting-paper suspended in buffer solutions at pH 5·2, 5·6, 6·0 and 7·0; it may therefore be concluded that over this range the virus of yellow fever is negatively charged.

In the case of blotting-paper immersed at pH 4·2 there was, however, no evidence that living virus had ascended the paper, for not only did the blotting-paper fail to produce any symptoms in the animals inoculated, but these monkeys were not immunized in any way, since on subsequent inoculation

with living yellow-fever virus they died from the disease after normal incubation periods.

It might, therefore, be concluded that the isoelectric point of yellow-fever virus lies between pH 4·2 and pH 5·2.

An alternative explanation may, however, be found, for in the case of vaccinia Douglas and Smith (1928) have shown that the virus is destroyed in an acid medium. Experiments were therefore carried out to determine whether yellow-fever virus is killed at pH 3·0 and 4·0. In one experiment infected liver that had been preserved on ice for 24 hours was ground up and suspended in buffer solutions of pH 4·2 and 5·2 respectively. After 4 hours suspensions were inoculated into monkeys; the virus had been killed at pH 4·2 but not at 5·2. In three further experiments with fresh virus, using both infected liver and blood-serum, the virus was found to survive exposure to solutions in the neighbourhood of pH 4·0 at room temperature for periods of 4 and 18 hours respectively, but was dead after 24 hours, whilst at the same room temperature the virus was killed after 4 and 18 hours respectively at pH 3·0.

It is evident, therefore, that yellow-fever virus is slowly destroyed at pH 4·0 and more rapidly at higher degrees of acidity.

CONCLUSIONS.

The virus of yellow fever is negatively charged at pH's ranging from 5·2 to 7·0. It is destroyed in acid media of pH 3·0 to 4·0.

REFERENCES.

Bedson, S. P., and Bland, J. O. W.—(1929) *Brit. J. Exp. Path.*, **10**, 67.
Brown, H. C., and Broom, J. C.—(1929) *Ibid.*, **10**, 219.
Douglas, S. R., and Smith, W.—(1928) *Ibid.*, **9**, 213.
Marchoux, Salimbeni and Simond.—(1903) *Ann. Inst. Pasteur*, **17**, 665.
Olitzky, P. K., and Boëz, L.—(1927) *J. Exp. Med.*, **45**, 685.

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CONTENTS.

EIDINOW, ALBERT.—THE EFFECT OF IRRADIATION ON COBRA VENOM AND ANTIVENIN 65

KELLAWAY, C. H.—THE ANAPHYLACTIC REACTION OF THE ISOLATED UTERUS OF THE RAT 72

GREEN, H. N., AND MELLANBY, E.—CAROTENE AND VITAMIN A: THE ANTI-INFECTIVE ACTION OF CAROTENE 81

ROBERTS, W. MORRELL.—VARIATIONS IN THE PHOSPHATASE ACTIVITY OF THE BLOOD IN DISEASE 90

DOUGLAS, S. R., AND SMITH, WILSON.—A STUDY OF VACCINAL IMMUNITY IN RABBITS BY MEANS OF *IN VITRO* METHODS 96

PIJPER, ADRIANUS, AND DAU, HELEN.—TYPHOID AGGLUTINATION AFTER ORAL IMMUNIZATION 112

LAWRENCE, R. D., MADDERS, KATE, AND MILLAR, H. R.—THE EXCRETION OF INSULIN IN URINE 117

MAITLAND, H. B., AND LAING, A. W.—EXPERIMENTS ON THE CULTIVATION OF VACCINIA VIRUS 119

FLEMING, ALEXANDER, AND MACLEAN, IAN H.—ON THE OCCURRENCE OF INFLUENZA BACILLI IN THE MOUTHS OF NORMAL PEOPLE 127

HINDLE, E., AND FINDLAY, G. M.—THE ELECTRICAL CHARGE OF YELLOW-FEVER VIRUS 134

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